CALCIUM INTAKE, VITAMIN D STATUS, PLACENTAL VITAMIN D RECEPTOR (VDR) EXPRESSION, PLACENTAL CALCIUM TRANSPORT, AND NEONATAL OUTCOMES IN ADOLESCENT PREGNANCY

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CALCIUM INTAKE, VITAMIN D STATUS, PLACENTAL VITAMIN D RECEPTOR (VDR) EXPRESSION, PLACENTAL CALCIUM TRANSPORT, AND NEONATAL OUTCOMES IN ADOLESCENT PREGNANCY

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Pregnancy and adolescence are both periods of elevated calcium (Ca) demand. Pregnancy during adolescence places both mother and fetus at risk for adverse skeletal outcomes. The overall goal of this research was to address how maternal Ca intake and vitamin D status impact calcitropic hormones and fetal bone development during pregnancy, and investigate how effects may be mediated at the level of the placental in a skeletally immature population.

Maternal Ca intake,25(OH)D status calcitropic hormones (PTH and $1,25(OH)_2D$) and fetal biometry measures (fetal femur and humerus length and birth length) were monitored across pregnancy in 171 pregnant adolescents (≤18 yrs). Neonatal hormonal status and placental tissue were obtained at delivery. Stable Ca isotopes were administered to twelve adolescents early in labor (^{44}Ca orally and ^{42}Ca intravenously) to obtain a dynamic measure of maternal-to-fetal Ca transfer.

Among these adolescents, 25(OH)D insufficiency (≤20 ng/mL) was prevalent (~50%) and PTH was inversely related to 25(OH)D status throughout pregnancy, and was elevated (≥60 pg/mL) in 24% of teens at term. Maternal 25(OH)D was also negatively associated with $1,25(OH)_2D$ concentrations. Associations with Ca intake were less evident, indicating that maternal vitamin D status is a key determinant of the calcitropic hormone response to pregnancy.

Maternal Ca intake and 25(OH)D status interacted to influence fetal femur and humerus Z-scores; sufficient status of one nutrient was associated with improved long bone Z-score when the other nutrient was limited. This interaction remained evident at delivery, and was associated with neonatal birth length.

At the level of the placenta, VDR expression was positively related to neonatal $1,25(OH)_2D$, and was higher in neonates with low 25(OH)D status. Placental VDR was also positively related to fetal femur length. Of note, placental VDR expression was a significant predictor of maternal-to-fetal ^{42}Ca transport which was itself significant in a model of fetal femur Z-score. Placental VDR expression was responsive to fetal endocrine signals and appeared to impact fetal skeletal growth via modulation of placental Ca transport. In a skeletally immature pregnant adolescent, achieving adequate 25(OH)D status and Ca intake may improve calcitropic hormone levels and optimize fetal skeletal accretion.

BIOGRAPHICAL SKETCH

Bridget Victoria Essley was born in Rochester, NY to Peter and Deborah Essley. She is the second child and has 3 wonderful siblings: Jason, Grace, and Claire. She grew up in West Irondequoit and attended Colgate University for her undergraduate degree. There, she majored in Molecular Biology and planned to attend medical school. After graduation, she worked for two years in the research laboratory of Dr. Manju Bhat at the Cleveland Clinic Foundation, where she decided that medical research was where she wanted to make a career. While in Cleveland, Bridget met David Young, whom she will marry 33 says after defending her doctoral dissertation. In the fall of 2007, she began her doctoral research at Cornell University in the Department of Nutritional Sciences. There, Bridget worked with Dr. Kimberly O'Brien in a lab focusing on micronutrient deficiencies during pregnancy and the impact on maternal and fetal health.

DEDICATION

This research is dedicated to the teens who participated in my studies, and their infants.

I will be forever grateful that they allowed me to share such a precious moment in their lives.

I have learned so much form these young women, and so often been inspired by them.

May God bless them.

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BMI	Body Mass Index (kg/m^2)
DRI	Dietary Reference Intake
RDA	Reference Daily Allowance
EAR	Estimated Average Requirement
AI	Adequate Intake
UL	Upper Limit
Ca	Calcium
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol
25(OH)D	25-hydroxyvitamin D; calcidiol
$1,25(OH)_2D$	1,25-dihydroxyvitamin D; calcitriol
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
DBP	Vitamin D Binding Protein
PTH	Parathyroid Hormone
BMD	Bone Mineral Density
BMC	Bone Mineral Content
LBW	Low Birth Weight (< 2500 g)
SGA	Small for Gestational Age
IUGR	Intraueterine Growth Restriction
LGA	Large for Gestational Age (> 4000 g)
FDIU	Fetal Death In-Utero
TRPV6	Transient Receptor Potential Vanilloid Receptor 6
Ca_{9DK}	Calbindin 9DK
Ca_{28DK}	Calbindin 28DK
NA	Natural Abundance

CHAPTER 1

INTRODUCTION

Specific Aims

Calcium (Ca) dynamics are dramatically altered during pregnancy to meet the Ca demands of the developing fetus. Maternal vitamin D status has also been increasingly linked to a variety of maternal and offspring health outcomes. This pregnancy-induced increase in Ca demand, and the importance of maintaining adequate vitamin D status is especially profound in the context of adolescent pregnancy when fetal skeletal development coincides with the demands and physiological stresses of adolescent growth. Additionally, adolescents often consume suboptimal Ca intakes and have insufficient vitamin D status, which may increase the risk of adverse maternal and fetal outcomes. Despite the number of studies linking maternal Ca intake and vitamin D status across gestation with maternal and offspring outcomes, the response of the calcitropic hormones, parathyroid hormone (PTH) and 1,25-dyhydroxyvitamin D ($1,25(OH)_2D$) during pregnancy, and how this response may be impacted by nutritional status are not well understood in at-risk populations. There is also a lack of data addressing regulation of Ca partitioning during pregnancy at the level of the placenta. Further research is needed to characterize how calcitropic hormone and nutritional status impact placental gene expression, nutrient partitioning to the fetus, and fetal development.

The goals of this research were fourfold: 1) To characterize the temporal trends and determinants of the two calcitropic hormones across pregnancy in an adolescent population, and to describe relationships between these hormones and the impact of nutritional (Ca and vitamin D) status on these relationships; 2) To determine how maternal Ca intake and vitamin D status during pregnancy are associated with measures of fetal and neonatal skeletal outcomes; 3) To determine if vitamin D status or Ca intake is associated with placental vitamin D receptor (VDR) expression, and if variation in expression is correlated with fetal long bone length; and 4) To

obtain in-vivo measures of maternal-to-fetal Ca transfer, to describe determinants of this transfer *in-utero,* and determine if increased transfer is associated with improved fetal skeletal health, as assessed by long bone length.

The specific aims and hypotheses are:

I. To characterize the temporal trends in calcitropic hormones, PTH and 1,25$(OH)_2$D during pregnancy and elucidate relationships between these hormones and Ca intake and 25-hydroxyvitamin D (25(OH)D) status in the pregnant adolescent and her neonate.

Hypotheses: 1) PTH will increase across pregnancy as a compensatory response to improve Ca availability in order to satisfy fetal Ca demands. 2) Maternal vitamin D status will be associated with PTH and 1,25$(OH)_2$D in pregnant adolescents.

II. To determine if maternal Ca intake and vitamin D status during pregnancy are related to measures of fetal and neonatal bone health obtained *in-utero* and at birth, and to investigate potential interactions between maternal Ca intake and vitamin D status on fetal and neonatal outcomes.

Hypothesis: Maternal Ca intake and vitamin D status will both be positively correlated with fetal femur and humerus length Z-scores and birth length. An interaction between these nutrients will be relevant, such that achieving sufficiency of one nutrient has the most significant impact when the other nutrient is suboptimal.

III. To determine if vitamin D status and Ca intake are associated with placental VDR expression and investigate relationships between placental VDR expression and fetal femur length Z-scores.

Hypotheses: 1) Placental VDR expression will be increased when 25(OH)D is low as a compensatory response to increase placental capacity for vitamin D activity. 2) Placental VDR expression will be positively associated with fetal femur Z-scores.

IV. To obtain in-vivo measures of placental Ca transfer *in-utero* using stable Ca isotopes and to characterize determinants of this transfer and investigate if increased transfer is associated with longer fetal femur and humerus lengths.

Hypothesis: Placental Ca transfer will be positively associated with placental VDR expression, with maternal 25(OH)D, and with fetal long bone (femur and humerus) length.

Together these data will improve our understanding of the calcitropic response to pregnancy in pregnant adolescents and the impact of inadequate vitamin D and Ca intake on fetal and neonatal bone outcomes. The data provided will inform dietary recommendations provided to this group.

Background and Significance

The United States has the highest adolescent pregnancy rate of any industrialized nation (1); approximately 410,000 adolescents (≤ 19 years) gave birth in 2009 (2). During pregnancy, maternal Ca intake must meet both maternal demands and those of the developing fetus. Over the course of gestation, approximately 30 g of Ca are transported across the placenta and incorporated into the fetal skeleton (3). Placental Ca flux markedly increases in the third trimester, when ~80% of net Ca transfer occurs (4). Peak rates of fetal Ca deposition occur at 35 weeks gestation and are thought to approximate 330 mg/d (5). This pregnancy associated increase in Ca demand is especially profound in the context of adolescent pregnancy, when fetal development occurs in an environment that is already physiologically stressed due to the additional Ca demands of adolescent growth.

Maternal physiology does respond to the Ca demands of pregnancy by increasing intestinal Ca absorption and metabolic bone activity (6). In spite of these adaptations, many studies of bone turnover during pregnancy have detected a net loss of maternal bone across gestation (7-9). This loss has been reported to be more severe among pregnant adolescents than adult women (10;11). As the majority of Ca that crosses the placenta is incorporated into the developing fetal skeleton, insufficient Ca intakes and/or an insufficient physiological adaptations during pregnancy may result in adverse fetal outcomes including decreased fetal skeletal growth and bone mineralization (11-15).

Vitamin D status plays an equally important role in the maintenance of Ca homeostasis during pregnancy due to its role in the regulation of Ca homeostasis. Vitamin D status has been linked to a variety of both maternal and fetal outcomes (16). The current Ca intake recommendations for pregnant adolescents (≤ 18 years) do not differ from their non-pregnant

peers (EAR = 1100 mg/d) (17). However, the adolescent EAR/RDA is 300 mg higher than the EAR/RDA for pregnant and non pregnant adult women ≥ 19 years of age (17). **Table 1.1** presents the 2011 DRI's for Ca and vitamin D for pregnant and non-pregnant adolescents and adult women.

Table 1.1.

Calcium and Vitamin D DRI Recommendations for Pregnant and Non-pregnant Adolescents and Adult Women (17)

		Ca (mg/day)			**Vitamin D (IU/day)**		
Stage	**Age (years)**	EAR	RDA	UL	EAR	RDA	UL
Non-pregnant	9 - 18	1100	1300	3000	400	600	4000
Pregnant	14 - 18	1100	1300	3000	400	600	4000
Non-pregnant	19 - 30	800	1000	2500	400	600	4000
Pregnant	19 - 50	800	1000	2500	400	600	4000

EAR – Estimated Average Requirement
RDA = Reference Daily Allowance
UL = Upper Limit

The 2010 IOM committee on Ca and vitamin D intake recommendations identified gaps in knowledge when data were lacking. The committee reported that research is needed to provide data regarding the physiology of the metabolism of Ca and vitamin D as well as data detailing the interactive and independent effects of Ca and vitamin D (17). Data from this dissertation research will contribute to these research needs to fill current knowledge gaps and inform future

studies and interventions designed to reduce adverse maternal and fetal health outcomes in adolescent pregnancy.

I. Calcium Metabolism and Vitamin D Endocrinology

Regulation of Ca Homeostasis

Calcium homeostasis is tightly regulated in the body at the level of the intestine, bone, and kidney. In the intestines, Ca is absorbed paracellularly via concentration gradient-driven diffusion (18). Calcium can also be actively absorbed by several Ca-transport proteins, including transient receptor potential vanilloid 6 (TRPV6), which imports Ca from the gut lumen across the brush border membrane of enterocytes. Once in the enterocyte, Ca is shuttled across the cell by Ca-chaperone proteins such as calbindin 9DK and 28K, and actively exported into circulation by Ca export proteins, such as the plasma membrane Ca ATPase's (PMCA's), located on the basolateral membrane (19). A more detailed discussion of these proteins is located in **Section V**. Active absorption increases the fractional absorption of dietary Ca above that which results from passive diffusion alone. It is this active absorption mechanism that is hormonally regulated by vitamin D. Active absorption is commonly observed to be inversely related to Ca intake. Active Ca absorption is upregulated when Ca intake is low (< 400 mg/day), and can account for up to 80% of total absorbed Ca (20). Conversely, active Ca absorption plays a diminished role when Ca intake is high. In the context of high habitual Ca intake (~ 2000 mg/day), paracellular absorption is adequate to meet Ca demands and active absorption contributes less than 10% of net Ca absorption (18).

Calcium homeostasis is tightly regulated by three main hormones: $1,25(OH)_2D$ (also known as calcitriol), PTH, and calcitonin. In humans, vitamin D is a unique micronutrient in that

it is both obtained from the diet and endogenously produced. Both vitamin D_3 (cholcalciferol) and vitamin D_2 (ergocalciferol: from plant sources) can be obtained from the diet (21). The majority of daily vitamin D requirements are thought to be met by endogenous production of vitamin D_3, which is produced in the skin from 7-dehydrocholesterol upon exposure to UVB light (22). Once released into circulation from the skin or via the lymphatic system after digestion, the vitamin D binding protein (DBP) transports vitamin D_2 and vitamin D_3, as well as their downstream metabolites (23).

Calcitriol is produced by successive hydroxylations of vitamin D_2/D_3 first in the liver and then in the kidneys (18). In the liver, vitamin D_2 and D_3 are hydroxylated by the 25-hydroxylase enzyme (CYP2R1) to become 25(OH)D (24). Because production of 25(OH)D (calcidiol) in the liver is not regulated, circulating concentrations of 25(OH)D reflect both dietary intake and endogenous production. As such, circulating concentrations of 25(OH)D serve as an acceptable biomarker of vitamin D status (25). In the kidneys, the 1α-hydroxylase (CYP27B1) further hydroxylates 25(OH)D generating $1,25(OH)_2D$ (24). Serum $1,25(OH)_2D$ is the active hormonal form of vitamin D. As such, the activity of the 1α-hydroxylase and net production of $1,25(OH)_2D$ remains tightly regulated. The inactive metabolite, $24,25(OH)_2D$, can also be produced from 25(OH)D in the kidney, by the 24-hydroxylase (CYP24A1) (18). Increased concentrations of $1,25(OH)_2D$ serve as negative feedback for its own production, by decreasing $1,25(OH)_2D$ production and increasing $24,25(OH)_2D$ production.

The majority of circulating $1,25(OH)_2D$ is bound to DBP which has a higher affinity for 25(OH)D, and thus leaves a small fraction of $1,25(OH)_2D$ free while the majority is bound (26). It is this free $1,25(OH)_2D$ that is hormonally active. Once released into the circulation, free-$1,25(OH)_2D$ can bind cell surface receptors and initiate various non-genomic rapid responses

within the cell that have many downstream effects including increased cellular Ca^{2+} concentrations and pH (27). Calcitriol also elicits genomic responses by binding to its receptor, the vitamin D receptor (VDR) and migrating to the nucleus. This complex ($1,25(OH)_2D$ / VDR) forms a heterodimer with the retinoid X receptor (RXR) and then binds to specific DNA sequences referred to as vitamin D response elements (VDRE's) (27). Upon binding VDRE sequences, this complex initiates the co-localization of additional regulatory elements, ultimately resulting in increased or decreased transcription of VDRE-containing genes. This is the mechanism whereby $1,25(OH)_2D$ increases the transcription of many Ca-transport proteins in the gut, thus increasing active Ca absorption (3;18).

This mechanism of $1,25(OH)_2D$ mediated regulation of VDRE containing genes is also vital to bone growth, mineralization, and maintenance of bone health (28). Additional roles of vitamin D activity, outside of the classically described role in Ca homeostasis, have been extensively explored in recent years. In addition to bone and small intestine, VDR has been detected in over 30 tissues in the human body, including placenta (21). Recently, the role of $1,25(OH)_2D$ in the induction of the innate immune system has been described in detail (29;30). Vitamin D may also play a role in the regulation and function of the adaptive immune system, minimizing inflammation and autoimmune disease (31;32). Calcitriol also regulates genes that are responsible for cell proliferation, differentiation, and apoptosis (24). Epidemiological studies have documented several relationships between low vitamin D status and chronic diseases and infections/inflammatory states including type I and II diabetes, bacterial vaginosis, preeclampsia, breast, prostate, and colorectal cancers, and all-cancer mortality (33-41). Thus, $1,25(OH)_2D$ plays a role in the regulation of hundreds of genes and many physiological processes, beyond those simply related to Ca metabolism and bone health. However, it should be noted that the

recent (2010) IOM committee for establishing the vitamin D and Ca DRI's found that there was insufficient evidence to conclude a causal relationship or dose response for any other health outcome related to vitamin D other than bone health (17). More research is clearly needed to detail these relationships and increase our understanding of all physiological consequences of vitamin D deficiency.

In light of the far-reaching effects of $1,25(OH)_2D$, it becomes clear why circulating concentrations are so tightly regulated. Parathyroid hormone is one of primary endocrine controls regulating production of $1,25(OH)_2D$. In the parathyroid gland, PTH secretion is coupled to membrane Ca^{2+} sensing receptors, which ensure PTH is secreted only under conditions of low serum ionoized Ca^{2+} (18). Upon secretion, PTH exerts direct effects on bone and kidneys, increasing resorption of bone and decreasing urinary Ca excretion. Both of these short term effects increase serum Ca concentrations to maintain homeostatic serum Ca levels (3). In the kidneys, PTH also acts to increase 1α-hydroxylase activity which increases net renal conversion of 25(OH)D to $1,25(OH)_2D$ when Ca and/or phosphorus status is low (42). Thus, in non-pregnant adults, PTH and $1,25(OH)_2D$ concentrations are inversely related when Ca economy is strained (potentially resulting from inadequate Ca intake or low vitamin D status) (42). Due to its stimulation of $1,25(OH)_2D$, PTH is also indirectly associated with intestinal Ca absorption. In serum, PTH has both short term and chronic effects on whole body Ca homeostasis. As part of a negative feedback loop, increased $1,25(OH)_2D$ concentrations inhibit PTH secretion, assuring that Ca balance is maintained. Under conditions of chronic vitamin D or Ca insufficiency, PTH levels can remain elevated to maintain continued synthesis of $1,25(OH)_2D$. This persistent elevation in PTH as a result of low vitamin D or Ca status is referred to as secondary hyperparathyroidism, and may have detrimental long-term effects on

bone health (43). **Figure 1.1** below diagrams the integrated roles of 25(OH)D, PTH, and $1,25(OH)_2D$ in the regulation of Ca homeostasis.

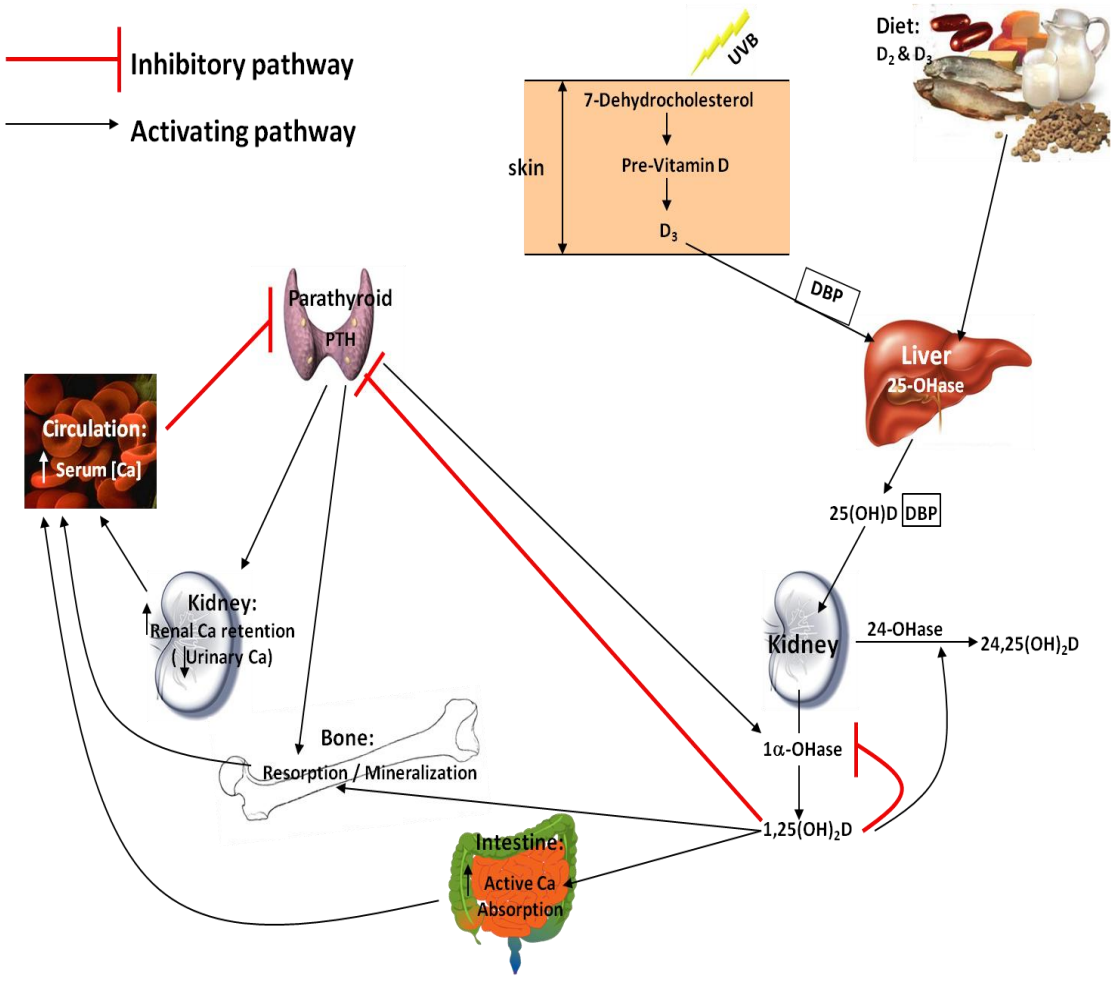

Figure 1.1. **Diagram of 25(OH)D, PTH, and $1,25(OH)_2D$ Regulation of Serum Calcium** Serum Ca is tightly regulated by PTH and vitamin D. Black arrows indicate stimulatory or activating pathways, and red lines indicate inhibitory pathways. Calcitriol ($1,25(OH)_2D$) stimulates active Ca absorption and bone resorption and PTH stimulates bone resorption and renal Ca conservation. Calcitriol synthesis is tightly regulated and $1,25(OH)_2D$ serves as negative feedback inhibitor of its own production in the kidney and also decreases PTH secretion.

Not detailed in **Figure 1.1** are the integrative effects of phosphorus (P) on Ca homeostasis, and vice versa. Phosphorus is the second largest mineral component of bone, as a major component of the crystal hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) which is laid down in the inorganic matrix of bone during the mineralization process. Calcitriol synthesis is stimulated by low P concentrations, and serves to increase intestinal absorption of both Ca and P, and also increases release of both Ca and P from bone (44). Parathyroid hormone inhibits P reabsorption in the renal proximal tubules (45). Fibroblast growth factor 23 (FGF23) is a key regulator of P homeostasis discovered in 2000 (45). FGF23 is released into serum by osteocytes and osteoblasts when P is high. While FGF's normally exert local paracrine or autocrine effects, FGF23 regulates P homeostasis using systemic endocrine mechanisms. FGF23 has a similar impact on renal P reabsorption as PTH. FGF23 also inhibits the production of $1,25(OH)_2D$ in the kidneys (44) which decreases P absorption in the gut. In a negative feedback loop, increases in $1,25(OH)_2D$ cause an increase in circulating FGF23 (44), which contains a VDRE in its promoter (45). FGF23 also decreases secretion of PTH from the parathyroid gland (44). In this manner, P and Ca homeostasis are inter-connected. The role of FGF23 in calcitropic hormone status is a developing area of research.

Calcitonin is a hormone produced in the parafollicular cells of the thyroid and serves to oppose the actions of PTH when serum Ca is high. Thus, when serum Ca is high, calcitonin causes an increase in bone deposition, which reduces serum Ca levels to normal (46). Calcitonin is thought to play a very limited role in the physiological response to pregnancy (3;6)

Vitamin D Economy: Racial and Environmental Disparities

Endogenous vitamin D_3 production is influenced by many factors. Because cutaneous production can only occur when the skin is exposed to UVB between 290 – 315 nm, geographic

location, season of the year, pollution, and cloud cover all impact the amount and/or quality of UVB that reaches the ground-surface, thereby effecting capacity for vitamin D production (22;24;47;48). In northern latitudes > 42^0 (Boston, MA), no endogenous vitamin D_3 production occurs between the months of November through February due to the angle of the earth during this season (49). It should be noted that the study location of this research, Rochester, NY lies at 42^0 N.

Individual variation in skin pigmentation also affects dermal production of vitamin D. The concentration of melanin in the skin is responsible for the degree of skin pigmentation. Melanin absorbs UVB light of the same wavelength that is necessary to convert 7-dehydrocholesterol into cholcalciferol (22). In effect, melanin limits the amount of UVB available to produce vitamin D. As a result of this competition, dark skinned individuals may need upwards of six times more sun exposure to produce equivalent increases in circulating 25(OH)D observed in light skinned counterparts (50). For this reason, dark-skinned populations are at increased risk for lower circulating 25(OH)D and a higher risk of vitamin D deficiency than Caucasians residing in the same environment (43;47;48). Additionally, African Americans have higher average circulating concentrations of PTH, and a higher prevalence of secondary hyperparathyroidism compared to Caucasians (43;47;48;50). As a result, the inverse relationship between PTH and 25(OH)D (indicative of suboptimal 25(OH)D status) is more commonly detected in African American compared to Caucasian populations (22;47;51). Whether these differences in vitamin D status translate into differences in circulating levels of $1,25(OH)_2D$ remains controversial. It has been reported that $1,25(OH)_2D$ concentrations in African Americans are both comparable to (22;47) and higher than Caucasians (43;52-54). Few data exist regarding these possible differences during pregnancy.

Paradoxically, even though African Americans have lower vitamin D status and higher PTH, they have significantly higher skeletal bone mineral density (BMD) than Caucasians at every age, averaging between 11-17% higher BMD and bone mass (43;55). African American women (<50 years) display similar BMD to that of Caucasian men of the same age (55). African Americans also have a lower risk of osteoporosis than Caucasians, despite elevated PTH and lower 25(OH)D (43;55;56). When dietary Ca intake is changed from high to low Ca content, PTH, $1,25(OH)_2D$, and fractional Ca retention increase similarly in both African American and Caucasian women, indicating that the PTH/vitamin D axis regulates Ca homeostasis similarly in both races (53). However, when given $1,25(OH)_2D$ extraneously, the increase in fractional Ca absorption is blunted in African American women compared to Caucasian women, indicating a decreased "gut-sensitivity" to the PTH/$1,25(OH)_2D$ regulation of Ca absorption (52). In a cohort of younger girls (5-16 years) fractional absorption of Ca was more closely related to Ca intake in Caucasians than African American girls, confirming that the PTH and $1,25(OH)_2D$ axis may have a more prominent effect on the gut in Caucasians (57). Another source of the racial skeletal differences observed in adults may be that African Americans also seem to exhibit a decreased skeletal sensitivity to PTH, as bone mass and BMD are higher, and markers of bone resorption are lower in this racial group despite elevated PTH (43;58). However, African Americans exhibit an increased renal response to PTH to conserve Ca, as urinary excretion of Ca is lower in African Americans than Caucasians. This is observed in children and adults (43;53;57;59). Thus, while the same endocrinology regulates Ca homeostasis in both African Americans and Caucasians, the subtleties of this regulation may differ by race, potentially resulting from an evolutionary adaptation to dark skin's limited ability to endogenously produce vitamin D. When racial differences are observed in a vitamin D or Ca related outcome, it is difficult to assess

whether genetic differences or differences in nutritional status (namely 25(OH)D and related metabolites) are driving the results observed.

In addition to physical environment, skin pigmentation and diet, other factors also impact individual vitamin D status. Cultural and personal practices such as limited outdoor activity, extensive skin covering (such as veiling) or extensive sunscreen usage can place certain groups at elevated risk for inadequate vitamin D. Additionally, increased adiposity is a risk factor for insufficient vitamin D status (60). Various measures of adiposity including BMI, fat mass, leptin, percent body fat, visceral adiposity, and hip circumference are commonly found to be negatively associated with circulating 25(OH)D in pediatric, adolescent, and adult populations, regardless of race (61-66). As vitamin D is a fat soluble vitamin, it is postulated that vitamin D sequestration in fat compartments results in decreased bioavailability of vitamin D from dietary and endogenous sources in overweight and obese individuals (60). In all of these ways, vitamin D is a unique micronutrient as it is impacted by multiple modifiable and non-modifiable factors in addition to diet.

Diagnostic values for vitamin D deficiency and insufficiency vary in the literature and range from ≤ 10 to ≤ 30 ng/mL. In this research, vitamin D deficiency is defined as serum 25(OH)D ≤ 10 ng/mL. Many have argued for a more liberal cutoff, suggesting that 25(OH)D $\leq$ 20 ng/mL should be considered deficient (67). However, as the DRI defined 25(OH)D > 20 ng/mL as "adequate", the cutoff of ≤ 20 ng/mL will be used herein as vitamin D insufficiency (17).

In American children (≤ 21 years), older age, winter season, high BMI, and African American race are all associated with low 25(OH)D (61). Even in southern states, studies have reported an 18% prevalence of vitamin D insufficiency amongst girls; this prevalence was higher

in African American girls (48). A study of African American girls age 12-14 years (n = 21) in New York City in the winter found 100% of participants to be vitamin D insufficient, and 43% of the population sampled to be severely deficient (25(OH)D ≤ 8 ng/mL) (51). From the NHANES III data, 14% of the American adolescent population had insufficient levels of vitamin D, and African American teens had a 20 times higher risk of deficiency (68). The 2011 DRI's for vitamin D derived an EAR of 400 IU/day and an RDA of 600 IU/day for all age groups between 1 and 70 years in order to maintain serum 25(OH)D at a minimum of 16 ng/mL (for the EAR) and 20 ng/mL (for the RDA), assuming no endogenous production (17) (**Table 1.1**). Despite these recommendations, vitamin D insufficiency remains a public health concern both in the United States and globally.

Ca Homeostasis in the Adolescent

Adolescence is a time of physiological strain. Nutritional demands increase to accommodate the physical growth that occurs at this time. Non-pregnant adolescent females have increased PTH, fractional Ca absorption, rates of bone turnover, and decreased urinary Ca excretion compared to adult women (69;70). These alterations in Ca homeostasis result in greater Ca retention by ~240 mg/day, an amount needed to meet the Ca demands for bone mineralization associated with adolescent growth (69). Peak bone acquisition occurs by ~17 years in females (71), but small increases in bone mass continue to accrue for several years after linear growth is complete (72;73).

During adolescence and this period of elevated bone acquisition, girls exhibit higher rates of fractional Ca absorption, decreased urinary and fecal Ca loss, and higher net Ca retention than young adult women, despite similar 25(OH)D, 1,25(OH)2D, and PTH concentrations (69;74). Serum PTH increases during adolescence to accommodate the elevated Ca demand and rates of

bone formation (75). Racial differences in calcitropic hormones and bone acquisition also become evident by adolescence, as African American teens exhibit higher $1,25(OH)_2D$ concentrations, fractional Ca absorption, and rates of bone deposition than Caucasian counterparts (57;59). These racial differences are consistent with the higher BMD, and lower osteoporosis risk that have been documented in African American adults (43;71). Pregnancy is also a physiological state of increased nutritional demands. Pregnancy during adolescence may place both the mother and her developing fetus at risk for inadequate nutrition and adverse outcomes.

Fetal Bone Development

There are two main types of bone development that occur *in-utero.* Intramembranous ossification is less common and involves direct ossification of membranous sheaths that derive from mesenchymal cells. This type of bone formation results in flat bones, such as those of the skull. The limb bones result from ossification of cartilaginous models in a process referred to as endochondral bone formation (76). This more complex process begins with the differentiation of embryonic mesoderm cells into mesenchyme, or embryonic connective tissue. Mesenchymal cells will condense at skeletal sites and differentiate into chondrocytes and form chondrification centers that form bone models (76;77). Cartilage first appears in the embryo by the fifth week of gestation (76). In long bone formation, chondrocytes at the center of the condensation halt proliferation and become hypertrophic. These hypertrophic cells direct adjacent cells to become osteoblasts, direct mineralization of the cartilaginous matrix, and attract blood vessels. The osteoblasts mineralize the cartilaginous matrix by laying down hydroxyapatite crystals, which provide bone strength and also serve as a reserve for Ca and P. The majority of fetal skeletal mineralization and Ca accretion (~80%) occurs in the third trimester of pregnancy when rates of

Ca transfer to the fetus peak (78). The central region of bone formation in the diaphyses (or shaft) of the developing long bone is called the primary ossification center, from which chondrocytes continue to proliferate, lengthening the bone. Near the time of birth, secondary ossification centers form at the ends of the long bone through similar cycles of chondrocyte proliferation and hypertrophy, vascularization and mineralization (77). These secondary ossification centers are the epiphysial growth plates, which remain active to facilitate the skeletal growth of childhood and finally fuse when adult height is achieved, by ~age 17 in females and later in males (79). Each of the individual steps of bone development are facilitated by a complex series of transcription factors and endocrine signals that regulate cell differentiation and recruitment of additional necessary cell types, bone formation, growth, and mineralization. Vitamin D, the calcitropic hormones, and serum Ca concentrations all contribute to the regulation of bone development and growth (80-82).

II. Ca Metabolism and Vitamin D Endocrinology During Pregnancy

Physiological Adaptations to Ca homeostasis during Pregnancy

Over the course of pregnancy, approximately 30 g of Ca is transferred from maternal circulation to the fetus (5). During pregnancy, maternal Ca homeostatic mechanisms are altered and others are introduced in order to accommodate the increased Ca demand. There are two main physiological alterations that occur during pregnancy to increase Ca availability to the fetus. Fractional Ca absorption from the gut increases early in pregnancy and is elevated upwards of two-fold at term (83-86). This increase in absorption, combined with plasma volume expansion increases maternal total exchangeable Ca pool size by 20% over the last five weeks of gestation (83). The large Ca transfer to the fetus occurs despite increased urinary Ca losses

which result from an increased plasma volume and glomerular filtration rate during pregnancy (13).

The second major adaptation that occurs to maintain Ca homeostasis during pregnancy is increased metabolic bone activity. Biochemical markers of bone turnover increase early in gestation and continue to rise throughout pregnancy, and are elevated upwards of 200% at term (8;9;84;87). Biochemical markers of bone resorption often increase earlier in gestation than markers of formation (8;9;13;84;88), and many studies have detected decreases in bone mineral density (BMD) and other indexes of bone quality at certain sites across pregnancy, especially in trabecular-rich bone (8;9;11;15;87;89). These losses of BMD observed in pregnancy are often recovered post-partum with the resumption of menses (13;15;87). However, no firm consensus exists regarding the skeletal site and permanence of pregnancy-induced bone loss.

Some studies postulate that the increases in Ca absorption are the primary source of fetal Ca supply, which is later supplemented by increases in bone Ca resorption (85). This is because the increase in fractional Ca absorption is offset by an increase in urinary Ca loss, and increases in absorption alone are insufficient to meet the demands of fetal development (8;83). In reality, the actual source of Ca supplied to the fetus is likely to vary by degree to which Ca absorption and bone turnover are modified during pregnancy. Factors that may impact Ca homeostasis include: maternal age, parity, diet, and nutrient and endocrine status (87).

Because of the physiological adaptations that occur during pregnancy, including increased fractional Ca absorption, and because studies have documented a lack of association (or even a protective effect) between parity and bone health in women, the Ca DRI for pregnant women does not differ from that of non-pregnant women (EAR: 800 mg/d; RDA: 1000 mg/d). Similarly, the Ca DRI for pregnant adolescents does not differ from that of their non-pregnant

peers (EAR: 1100 mg/d; RDA: 1300 mg/day) (**Table 1.1**) (17). While recommendation for pregnant vs. non-pregnant teens do not differ, pregnant adolescents were identified as a population in need of more research by the IOM 2010 Ca and vitamin D DRI committee (17).

Vitamin D and Calcitropic Hormones During Pregnancy

Like Ca, vitamin D plays an important role in fetal bone development and maternal bone Ca retention and regulates hundreds of genes containing vitamin D response elements (VDRE's). Thus, maintenance of adequate levels of vitamin D during pregnancy may be critical to maternal and fetal health. Recently, the IOM released new recommendations for vitamin D intake for all populations including pregnant women. The new vitamin D DRI is the same for all population groups between the ages of 1 and 70 years (EAR: 400 IU; RDA: 600 IU) (**Table 1.1**) (17). This recommendation represents an increase of up to 3-fold from the previous vitamin D DRI which was set at an AI of 200 IU/day (25). However, as previously mentioned the NIH roundtable addressing vitamin D research needs highlighted both pregnant women and adolescents as high priority populations in whom more research is needed (90). Maternal 25(OH)D concentrations remain relatively stable across pregnancy in spite of marked increases in plasma volume (84;91). However, many pregnant women do not receive adequate dietary vitamin D or sufficient sun exposure given that the prevalence of vitamin D insufficiency in pregnant women and in newborns is high across the globe, ranging from ~30 - 90 percent (92-98). **Table 1.2** below shows the global prevalence of vitamin D insufficiency (25(OH)D $\leq$ 20 ng/mL) and deficiency (as defined by the authors) in pregnant women.

Table 1.2.

Global Prevalence of Vitamin D Deficiency and Insufficiency in Pregnant Women

Location	n	Time of Assessment	Insufficient (%)	Deficient (%)	Assessment Method
France[2] (99)	80	Term		46%	In-house RIA with rat serum
Turkey[1] (100)	100	Term		82%	IDS RIA
Ireland[1] (92)	43	Mean of three trimesters	48 – 77%	14 – 23%	IDS ELISA
Melbourne, Australia[2] (101)	374	28-32 weeks		7.2%	IDS RIA
Pittsburgh, USA[c] (93)	400	Term		17.1%	IDS ELISA
Sydney, Australia[1] (98)	971	23-32 weeks	48.8%	15%	Nichols Advantage Assay
Finland[1] (94)	98	Mean: 1st trimester and 2 days post-partum	98.0%	70.7%	IDS ELISA
Southampton, UK[1] (95)	424	34 weeks	36.6%	5.9%	Diasorin RIA
Southampton, UK[1] (96)	198	34 ± 2 weeks	31%	18%	IDS RIA
Baltimore, USA[3] (97)	80	Third trimester	46.3%	21.5%	Diasorin RIA
Boston, USA[2] (102)	40	Term		50%	In-house RIA
Winnipeg, Canada[3] (103)	50	Term		46%	Diasorin RIA
Amsterdam, Netherlands[2] (104)	3730	12-14 weeks	44.4%	23.1%	IDS ELISA

[1] Deficiency defined by authors as: 25(OH)D ≤ 10 ng/mL.
[2] Deficiency defined by authors as: 25(OH)D ≤ 12 ng/mL.
[3] Deficiency defined by authors as: 25(OH)D ≤ 15 ng/mL.
IDS = Immunodiagnostic systems

The fetus is incapable of dermal 25(OH)D production, and is completely dependent on maternal supply of 25(OH)D. As such, maternal and fetal levels of 25(OH)D are highly correlated (23;98-100;102;103;105-108). However there are inconsistent reports as to whether

neonatal 25(OH)D concentrations are similar to (93;99;100;102), or significantly lower (by ~25%) than maternal concentrations (23;103;105-108). Serum 25(OH)D freely crosses the placenta both free and bound to DBP, but is more efficiently transferred when unbound (26;109).

PTH

The role of PTH during pregnancy is more controversial. Studies report that PTH is either unchanged, remains low, or even decreases throughout the course of pregnancy in healthy women (3;6;8;9;88;91;110). Two studies (n = 16 and n = 14) identified a trend for increased PTH across gestation in adult women, but this trend did not reach statistical significance (9;111). Additionally, some studies have observed an *increase* in PTH late in pregnancy, especially in women with low Ca intakes (13;91;112;113). Furthermore, elevated PTH (≥ 46 pg/mL) was detected among 13% of a cohort of 971 pregnant women in Australia, and this prevalence was higher amongst women with poor vitamin D status (98). Because of such observations, it has been postulated that PTH may increase as a compensatory response to cause an increase in renal Ca retention and bone resorption in women with inadequate Ca intakes, poor vitamin D status and/or excess Ca demand (6;87;114). This hypothesis is supported by the finding that PTH is higher in pregnant adolescents compared to adult counterparts (70), as pregnant adolescents may have a higher Ca demand compared to adult women due to the combination of the Ca demands of the fetus and of a biologically immature maternal skeleton. These observations together support the postulation that, when whole-body Ca economy is strained during pregnancy, increased PTH may serve as a compensatory response to meet fetal Ca needs (6). The determinants of PTH secretion (i.e. maternal age, race, vitamin D status and Ca intake, etc.) and role of PTH in Ca homeostasis during pregnancy still remain poorly characterized, especially in at-risk populations.

Unlike 25(OH)D, the fetus is capable of endogenous PTH synthesis, and is not dependent on maternal transfer of this hormone. Neonatal concentrations of PTH are much lower than maternal concentrations at parturition (106;107;115-117). Thus, the fetus is less impacted by maternal changes in PTH.

1,25(OH)$_2$D

In contrast to the reported lack of change in 25(OH)D and PTH concentrations across pregnancy, circulating concentrations of 1,25(OH)$_2$D as well as vitamin D binding protein (DBP) increase during pregnancy (16;23;118;119). However, the increase in 1,25(OH)$_2$D is more substantial than that of DBP resulting in a 37 – 69% increase in free-1,25(OH)$_2$D (23;118-120). Other studies that have assessed only total 1,25(OH)$_2$D all report increases in circulation during early pregnancy (84-86;91;111;112;121-126). Whether circulating 1,25(OH)$_2$D increases steadily across gestation (84;85;123), or increases early in pregnancy and then plateaus later in gestation remains controversial (91;111;121;122).

The increase in 1,25(OH)$_2$D is due to increases in renal and extra-renal (ie: placental) synthesis of 1,25(OH)$_2$D (21;117;127). The relative contributions of each of these tissues are uncertain (13;87). The limited change in PTH commonly observed across normal pregnancy indicates that an alternative stimulation of 1,25(OH)$_2$D production must exist during pregnancy. A prime candidate for this role is PTHrP (parathyroid hormone related peptide) (3;6;87). Parathyroid hormone related peptide is secreted by the placenta and a number of other maternal tissues, and circulating levels increase throughout the course of pregnancy (3;6;13;128). The amino terminus of PTHrP and PTH are homologous, and both bind one of the three PTH receptors (PTH1R) (3). Administration of PTHrP has been shown to cause an increase in plasma 1,25(OH)$_2$D, analogous to PTH (129). Serum PTHrP levels also correlate with markers of bone

turnover during pregnancy further suggesting a role in regulation of Ca homeostasis (128). In addition to PTHrP, several other hormones, mainly of placental origin, are increased during pregnancy - all of which may potentially play a role in the regulation of $1,25(OH)_2D$ synthesis. These include insulin-like growth factor-1 (IGF-1), estrogen, placental lactogen, placental growth hormone, and prolactin (6;13;87). Details of the specific roles of each of these hormones in $1,25(OH)_2D$ synthesis during pregnancy remains unknown. It has recently been highlighted that research is needed to determine how $1,25(OH)_2D$ is increased in maternal circulation during pregnancy, and the relative contributions of the kidney and placenta (16).

Regardless of the source, increased $1,25(OH)_2D$ is thought to be responsible for the increased intestinal Ca absorption that occurs during pregnancy from $1,25(OH)_2D$-mediated regulation of Ca transport in the intestine (13). However, reports of relationships between $1,25(OH)_2D$ and Ca absorption during pregnancy are inconsistent (85;126). Calcitriol has a stimulatory effect on bone resorption (24) and increases in $1,25(OH)_2D$ parallel the increases in bone turnover observed during pregnancy (6). In summary, the increase in $1,25(OH)_2D$ that occurs early in pregnancy is well-documented, but the stimulus for the increased $1,25(OH)_2D$ production, and the role of elevated $1,25(OH)_2D$ during pregnancy is not fully understood.

It is universally found that neonatal concentrations of $1,25(OH)_2D$ are lower than maternal concentrations at birth, and the two are often unrelated (106;107;115-117). Some studies have reported a positive relationship between maternal and neonatal $1,25(OH)_2D$ (107;116) while others report a lack of association (115).

The fetus it is not dependent on maternal supply of $1,25(OH)_2D$, as it is capable of synthesizing $1,25(OH)_2D$ (130) and fetally produced $1,25(OH)_2D$ regulates fetal Ca homeostasis independent of maternal $1,25(OH)_2D$ concentrations in animal models (131). Whether maternal

$1,25(OH)_2D$ even crosses the placenta is controversial. Calcitriol does not cross the rat placenta, but does cross the sheep and primate placenta (132). Studies in perfused human placenta have indicated that $1,25(OH)_2D$ is capable of crossing the placenta, and is more efficiently transferred when unbound to DBP (26). The placenta is also capable of synthesizing $1,25(OH)_2D$ (133), yet whether placental $1,25(OH)_2D$ contributes to circulating fetal levels remains unknown (134). In summary, while the fetus is solely dependent on maternal supply of 25(OH)D, it is capable of a certain degree of self-regulation of Ca homeostasis because neonatal PTH and $1,25(OH)_2D$ are largely independent of maternal concentrations.

Relationships among 25(OH)D, PTH, and $1,25(OH)_2D$

Relationships among 25(OH)D, PTH and $1,25(OH)_2D$ are well characterized in non-pregnant populations, but these relationships are less clear during pregnancy even though several large studies addressing these relationships during pregnancy have been published within the last month (135;136).

Whereas it has been reported that $1,25(OH)_2D$ is positively associated with 25(OH)D in a study of 40 pregnant women (123), a recent meta-analyses (2010) of data from 26 studies of pregnant women found no correlation between maternal 25(OH)D and $1,25(OH)_2D$ concentrations (125). A recent large study (n = 530) by Hollis et al. documented a significant biphasic relationship between 25(OH)D and $1,25(OH)_2D$, with $1,25(OH)_2D$ increasing as 25(OH)D increased up to 40 ng/mL, at which point $1,25(OH)_2D$ plateaued (135). The authors concluded that a 25(OH)D concentration of at least 40 ng/mL was necessary to support maximum $1,25(OH)_2D$ production in pregnant woman (135). This study was a large randomized control trial providing pregnant women with either 400, 2,000, or 4,000 IU vitamin D/day from ~13 weeks gestation to term. However the relationship between 25(OH)D and

$1,25(OH)_2D$ was observed among all treatment groups combined, and was thus observational in nature. It is still unclear how 25(OH)D may impact $1,25(OH)_2D$ when 25(OH)D remains low across gestation.

Both a positive (137) and negative (123) relationship between $1,25(OH)_2D$ and PTH have been reported during pregnancy in ethnic populations. There are also inconsistencies regarding the relationship between 25(OH)D and PTH during pregnancy. Many studies assessing the two fail to report a relationship (84-86;112;138). In the large randomized control trial referenced above (providing women with either 400, 2,000, or 4,000 IU vitamin D/day), no differences in PTH were observed between treatment groups despite significant differences in 25(OH)D (135). However, the intent-to-treat analyses of this study should be interpreted with care because the randomization process was compromised due to study design changes that were made in response to IRB concerns with the high dose supplementation arms (135). The authors did observe that PTH decreased as 25(OH)D increased in African American women (in all treatment groups) (135). A negative relationship between 25(OH)D and PTH has been reported in other smaller studies of pregnant women (113;123). Finally, a recent cross-sectional study of pregnant women between 11 and 13 weeks gestation (n = 1,016) found that PTH was lowest in the summer season, and was inversely associated with 25(OH)D in African American women with insufficient 25(OH)D (136). Variation in maternal Ca intake, vitamin D status, race, and age and interactions among these characteristics likely contributed to the variable findings between studies. Failure to account for these differences may by a source of the lack of consensus in the relationships reported.

The pregnancy induced physiological changes in Ca homeostasis are controlled by hormonal adjustments that accompany pregnancy. The calcitropic hormones are impacted by

vitamin D status and Ca intake during pregnancy, and increasing data link these two nutrients with maternal and neonatal health outcomes. However, details regarding temporal changes in calcitropic hormones and how Ca intake and 25(OH)D status may impact these changes during pregnancy remain relatively elusive.

III. Adolescent Pregnancy

Peak bone mass is established during adolescence and in females, this narrow window of bone consolidation is largely completed by the age of 17 years (71). A pregnancy during this period of bone acquisition represents nine months of lost opportunity to increase maternal bone mass, if fetal skeletal needs are prioritized over those required for maternal skeletal growth. The elevated rates of bone turnover during pregnancy, combined with a lower maternal total body bone mineral content (BMC) reserve may place pregnant teens at an elevated risk of bone loss during pregnancy. This may also impact her peak bone mass potential and increase her risk of osteoporosis later in life. These elevated Ca demands also place the developing fetus at risk for suboptimal Ca supply and skeletal development.

Adolescent Pregnancy - the Competition for Nutrients

Rates of adolescent childbearing in the United States are the highest of any industrialized nation; approximately 30% of American girls will become pregnant at least once before the age of 20 (1). Pregnant adolescents are at increased risk for multiple adverse birth outcomes. Pregnant teens are more than twice as likely to deliver a low birth weight (LBW) or preterm infant (139;140). Adolescents also exhibit rates of neonatal mortality three times higher than adult woman (> 19 yrs); approximately 14% of adolescent pregnancies end in miscarriage or stillbirth (139). Pregnant adolescents are also more likely to experience maternal morbitities,

and exhibit twice the maternal mortality rate of adult pregnant women in the United States (139;141).

Associations between adolescent pregnancy and adverse birth outcomes are confounded by the fact that pregnant adolescents often possess many biological and social factors that are independently associated with adverse birth outcomes such as low pre-pregnancy weight, poor pregnancy weight gain, low education, smoking, drug use, and poverty (139;142;143). Furthermore, adolescents are more likely to have inadequate access to prenatal care (142), to consume diets of poor quality (high in fat and sugar and low in micronutrients) (17;144-146), and to have a high prevalence of infections (sexually transmitted and bacterial) (142). Additionally, adolescent pregnancy disproportionately impacts minorities, who are already at increased risk for poor birth outcomes (139;147). African American neonates have a higher preterm and mortality rate compared to Caucasian neonates regardless of maternal age (148-150). African American pregnant adolescents have been shown to have high rates of anemia (upwards of 66%), with one third of adolescents exhibiting depletion of body iron by the third trimester in approximately (142;151). Together these factors further compromise maternal and fetal health and likely contribute additional risk to adolescent pregnancy in addition to maternal physiological immaturity.

Animal models of adolescent pregnancy have indicated differential nutrient partitioning between mother and fetus in biologically immature females, favoring growth of maternal body at the expense of placental (and thus fetal) growth. This partitioning is dependent on maternal nutritional and growth status, as opposed to chronological age (152-154). A model of adolescent pregnancy in rapidly growing ewes is characterized by excessive maternal weight gain, but shorter gestation, reduced numbers of placental cotyledons (a measure of placentation and

placental efficiency), smaller placentas and smaller fetuses (152;153). It has been postulated that in "still-growing mothers" maternal growth is prioritized over fetal needs, limiting placental growth and placental capacity to supply adequate nutrition for fetal growth and development, resulting in small placentas and fetuses and decreased gestation (153;155).

Studies in humans are lacking, but analogous trends and outcomes have been observed in human adolescent pregnancies. Nutritional and growth status of the adolescent, rather than age, seems to have the most dramatic impact on pregnancy outcome. Studies that have assessed maternal growth across pregnancy (via knee height and percent parental height achieved) in adolescents have established that still-growing adolescents gain more weight but deliver infants of lower birth weight than non-growing counterparts, indicating that weight gain was due to maternal verses fetal growth (156-160). Under these conditions when maternal tissue is prioritized, the placenta may not develop or function normally. This is supported by decreased placental blood flow (by Doppler ultrasound) and decreased micronutrient concentration in cord blood from still-growing pregnant adolescents (147;161). Diet and nutritional status also plays a role; high sugar diets have been associated with an increased risk of a small for gestational age (SGA) infant and reduced gestation in teen pregnancies (144;147). Inadequate supply of nutrients may occur in adolescent pregnancy when demand of maternal plus fetal growth is not met by a nutrient-dense diet. The resulting competition for nutrients between mother and fetus places both members of the maternal/fetal dyad at risk for adverse outcomes (141).

Ca Homeostasis in the Pregnant Adolescent

If an adolescent becomes pregnant before achieving skeletal maturity, and is not consuming a Ca replete diet, she may increase bone resorption to meet fetal needs, which places herself and her fetus at risk for adverse bone health outcomes. Even though non-pregnant

adolescents have higher rates of fractional Ca absorption than non-pregnant adult women (69), pregnant adolescents *are* able to further increase fractional Ca absorption (162) to achieve the same fractional absorption observed in adult pregnant women. However, adolescents do not increase their fractional Ca absorption beyond that observed among pregnant adult women (84;86;162). Pregnancy does seem to have a more severe impact on the Ca homeostasis of teens versus adult women. Among women with low Ca intake, pregnancy in teens resulted in a larger increase in PTH and larger decrease in urinary Ca than was seen in adult counterparts (70). These teens also exhibited smaller increases in markers of bone resorption, and decreases (compared to increases) in markers of deposition compared to mature women (70). Together these data suggest that adolescents may have higher Ca demands and be at increased risk of pregnancy-induced bone loss. This is supported by data from a longitudinal study assessing bone density across pregnancy (as measured by calcaneus ultrasound) that detected greater bone loss in pregnant adolescents compared to adult women (11). Bone loss was most pronounced in the still-growing adolescents in this cohort (11). A cross sectional study of white women age 20-35 found that age at first pregnancy < 20 years was significantly negatively associated with mid-distal radial bone mass (10). A study of African American adolescent mothers (n = 15) detected "low bone mass for chronological age" (z-scores < -2) in 29% of the cohort early post-partum, which may indicate that significant bone loss had occurred across gestation (162;163). Furthermore, adolescent females are also likely to consume suboptimal Ca intakes, and have insufficient 25(OH)D status, which further places them at risk for a strained Ca economy during pregnancy (17;97).

In summary, it appears that adolescents' calcitropic and physiological response to pregnancy is similar to that of adult women, and this may not be sufficient to maintain maternal skeletal accretion while fully supporting fetal skeletal mineralization and growth.

IV. Vitamin D and Ca Fetal Programming

It is now well known that the *in-utero* nutritional environment may "program" the fetal metabolic profile and gene expression, impacting long term risk of developing subsequent chronic diseases in adult life. The *in-utero* supply of both 25(OH)D and Ca have been linked to a variety of neonatal and adult outcomes.

Vitamin D insufficiency has been associated with suboptimal skeletal development which may be sustained into adult life (164). In Australia, maternal vitamin D deficiency during pregnancy was linked with shorter gestation and reduced knee-to-heel length in fetuses. This reduction in long-bone length remained significant when accounting for reduced gestation (101). Vitamin D deficiency was also shown to affect bone density, as vitamin D-deficient infants had lower bone mass/body weight ratios than replete neonates in a Canadian study (103). In Korea, neonatal 25(OH)D was found to correlate positively with total body BMC. Infants born in winter months had lower 25(OH)D and 8% lower total BMC than summer born infants (165). In Great Britain, 3D ultrasound was utilized to show that decreasing maternal 25(OH)D status during pregnancy was associated with increased fetal femoral splaying indices (commonly seen in childhood rickets), as early as 19 weeks gestation (95). Infants born to Finnish mothers with mean 25(OH)D > 17.0 ng/mL (the median for the cohort) exhibited larger tibial BMC and cross sectional area than those born to mothers with 25(OH)D below 17.0 ng/mL (94). In summary, maternal vitamin D deficiency has been identified as a driving factor of the BMC of the fetal

skeleton (165). This effect of vitamin D status on fetal skeletal growth and mineralization suggests that vitamin D status *in-utero* may "program" that fetus for less-than-optimal peak BMD, and subsequent adverse bone health outcomes into adulthood.

In the same Finnish cohort mentioned above, 87 of the original 124 infants (70%) were assessed at 14 months. Despite 25(OH)D repletion and similar 25(OH)D observed in all infants by 14 months, the bone differences observed at birth were only partially ameliorated, suggesting that fetal 25(OH)D exposure *in-utero* had lasting impact on offspring bone cross-sectional area (166). Similarly, guinea pig pups born to vitamin D deficient mothers exhibit reduced body weight, length, and whole body and tibial BMC compared to pups born to sufficient sows (167). Moreover, after 28 days of supplemental vitamin D, this deficit remained (167). This suggests modulation of bone accrual *in-utero* may persist, affecting adult risk of osteoporosis (168). Research on an epidemiological scale has supported this theory. Offspring of women with low 25(OH)D late in pregnancy exhibit reduced whole body and lumbar spine BMC at nine years compared to offspring born to women with adequate levels of 25(OH)D during pregnancy (96). Similar results were found when using UVB exposure in the third trimester as a proxy for vitamin D status (96). Estimated ultraviolet exposure of women during their third trimester of pregnancy (based on historical meteorological data) has also been associated with the lean body mass, BMC, bone area, and BMD of offspring at nine years. From this data, it was estimated that every standard deviation increase in maternal UVB exposure of a white pregnant woman, was associated with a 5% decrease in lifetime fracture risk of that child (169). These data suggest that vitamin D status has the potential to effect fetal health long after the perinatal period, into childhood and adulthood.

More recently, deficient maternal vitamin D status and/or intake during pregnancy has also been linked to numerous other adverse neonatal and child health outcomes, in addition to bone health. These include reduced birth weight, SGA, asthma, respiratory problems, autoimmune disorders, and schizophrenia (170-178). These associations are controversial, and the recent IOM committee on Ca and vitamin D intake concluded that there was insufficient evidence to establish causality in these proposed programming effects of vitamin D (17). The need for more research in this area has been highlighted (16).

An insufficient Ca intake during pregnancy may result in reduced supply of Ca to the fetus and may also potentially "program" that fetus for suboptimal bone health later in life. In a controlled trial of disadvantaged Indian women (n = 87), those supplemented with Ca (300 or 600 mg/day) from week 20 of gestation through parturition, gave birth to neonates with significantly higher bone density than neonates born to women receiving placebo (179). In a supplementation trial of healthy American mothers (n = 256) randomized to receive 2 g/day Ca, or placebo from ≤ 22 weeks through delivery, supplementation in women with low initial Ca intake (< 600 mg/day) resulted in neonates with significantly higher total body, and lumbar spine BMC (180). After adjusting for maternal age and BMI, maternal Ca intake (diet plus supplement, accounting for adherence) was significantly associated with higher infant total body BMC (180). Dairy intake seems to have a similar impact (in countries that fortify milk with vitamin D, such as the United States) due to its high Ca and vitamin D content. In a supplementation trial of pregnant Caucasian adolescents, teens randomized to consume four servings of dairy per day gave birth to infants with higher 25(OH)D status and larger birth weights (by ~230 g) than observed in the Ca-only supplement group or the control group (181). Infants in the "dairy" group had 17% higher total body Ca than those in the control group (181).

Dairy intake has also been found to impact fetal bone growth as well; low dairy intake (< 2 servings per day) was associated with shorter fetal femur length in pregnant adolescents (12). These effects of maternal dietary intake may have a lasting impact on offspring bone health as maternal milk intake during pregnancy has been positively associated with offspring spinal BMD at 16 years (182). The high Ca demand of adolescence and pregnancy combined with the poor dietary quality documented in teens place their offspring at increased risk for these adverse bone and long term health outcomes.

V. Placental Expression of Ca-Related Proteins & VDR

Fetomaternal Interface - the Placenta

Humans have a hemochorial placenta; placental invasion of the uterine wall is extensive and maternal blood is separated from fetal circulation only by the fetal endothelium lining the allontoic capillaries, the fetal chorioallantoic mesoderm and the fetal chorionic epithelium. The syncytiotrophoblast forms a single multinucleated cell layer that surrounds the chorionic villi so that all nutrients must traverse the syncytiotrophoblast when crossing from maternal to fetal circulation. The apical membrane of the syncytiotrophoblast is microvillus, to increase the surface area for absorption – very similar to the intestinal enterocyte. The apical membrane is in contact with maternal blood, and the basal membrane directly faces the endothelial wall of the fetal capillary.

Throughout the course of pregnancy, the fetus is hypercalcemic reletive to maternal circulation; thus the transfer of Ca occurs against a concentration gradient (183). Ca flux to the fetus consists of three distinct processes within the placenta, each of which are regulated: 1) apical Ca entry from maternal circulation into the syncytiotrophoblast; 2) cytoplasmic transfer

across the syncytiotrophoblast to the basal membrane; and 3) basal efflux out of the syncytiotrophoblast into fetal circulation. Specific Ca-transport proteins identified in the placenta contribute to each of these processes, and 1,25$(OH)_2$D regulates expression of many of these transporters. **Figure 1.2** below shows the cellular layers and proteins involved in this transport.

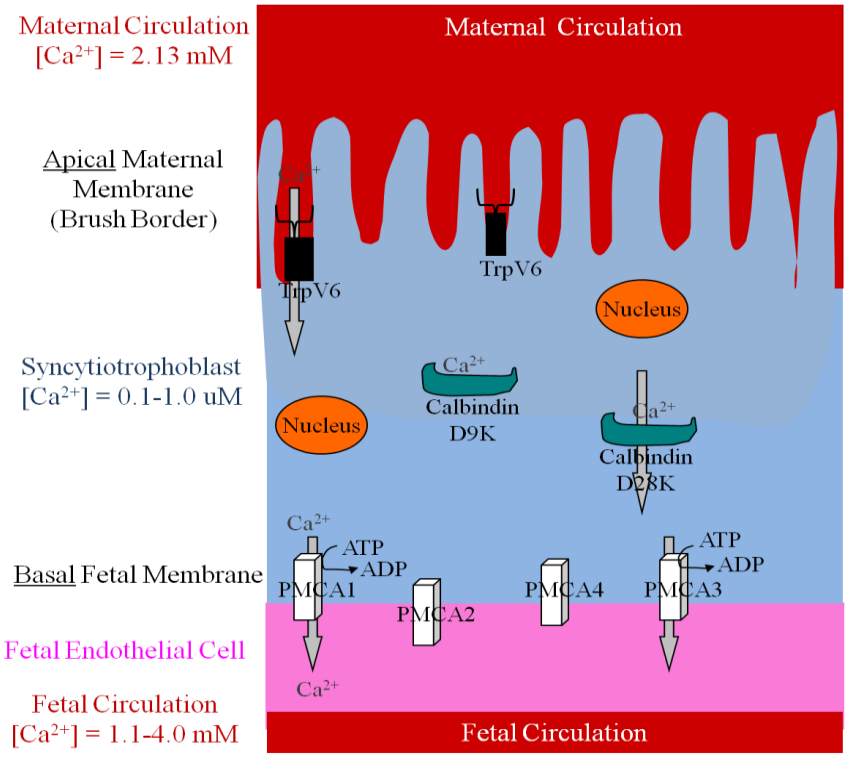

Figure 1.2 Placental Transport of Calcium from Maternal to Fetal Circulation
The fetus is maintained hyercalcemic to the mother and Ca is transported against a concentration gradient across the syncytiotrophoblast by three distinct regulated steps: 1) apical entry, 2) cytoplasmic transfer, and 3) basal efflux. Several Ca transport proteins play a role in each of these steps. The transient receptor potential vanilloid 6 (TRPV6) imports Ca from maternal circulation. Calbindin 9DK and 28K shuttle Ca across the syncytiotrophoblast, and plasma membrane Ca ATPase 1-4 export Ca into fetal circulation.

The TRPV6 channel is a voltage-gated Ca channel expressed on the apical membrane of the enterocyte and the syncytiotrophoblast, where it plays a pivotal role in facilitated Ca entry

into the cell (183). Interestingly, the TRPV6 gene contains multiple VDRE's, and thus expression of the Ca channel is regulated by availability of $1,25(OH)_2D$ (27;184). In Caco-2 cells (intestinal cell line) treatment with $1,25(OH)_2D$ for eight hours results in a 60 fold increase in TRPV6 mRNA (185). Treatment with 25(OH)D also results in an increase in TRPV6 expression in cells that express the 1α-hydroxylase (which includes the placenta (186)) indicating the capacity for both intracrine and endocrine regulation of TRPV6 expression by $1,25(OH)_2D$ (185).

Calbindin D9K ($CaBP_{9K}$) and Calbindin D28K ($CaBP_{28K}$) are two Ca transport proteins that shuttle Ca across the cytoplasm of the enterocyte (18). Both of these proteins are detected in the human placenta and expression is high in the cytosol of the syncytiotrophoblast (110;187). These proteins are believed to play similar roles in placental Ca transport, as expression of these proteins increases in late gestation (188) when rapid fetal bone growth occurs (183). Furthermore, human syncytiotrophoblasts in culture exhibit an time-dependent increase in $CaBP_{28K}$ RNA that parallels rates of Ca uptake (187). Both the $CaBP_{9K}$ and $CaBP_{28K}$ genes contain VDRE's, and their expression is increased by $1,25(OH)_2D$ in placental cells via the VDR-mediated pathway (18;183;189-191). At the basal membrane, Ca is transported out of the syncytiotrophoblast in an energy dependent manner. Four plasma membrane Ca ATPases (PMCA 1-4) all export Ca (183). Expression of these proteins increases late in gestation when Ca flux increases (183;192). The PMCA1 and 4 are expressed at higher levels than PMCA2/3, and it is thought that PMCA1/4 are the main modulators of Ca efflux to fetal circulation.

Both 25(OH)D and $1,25(OH)_2D$ contribute to regulation of each step of placental Ca transport. As such, vitamin D status and placental VDR expression may regulate placental Ca

transport, which provides a potential mechanism for the programming effects attributed to deficient vitamin D status *in-utero*.

VDR and other Vitamin D Machinery in the Placenta

The placenta expresses both the 1α-hydroxylase enzyme and the VDR (186). Both of these proteins are expressed in the syncytiotrophoblast and are co-localized in placental tissue (193-195). This indicates the potential for paracrine and intracrine vitamin D activity within the placenta. Indeed, endogenous placental production of $1,25(OH)_2D$ has been demonstrated in-vitro (133;196). Furthermore, $1,25(OH)_2D$ increases both the expression of VDR (189) and the vitamin D 24-hydroxylase enzyme, and down-regulates the expression of the 1α-hydroxylase gene in the placenta (197). These observations indicate a tightly controlled regulation of paracrine vitamin D activity at the level of the placenta. Additionally, it was recently discovered that the vitamin D 24-hydroxylase gene is fully methylated in the placenta, resulting in decreased placental CYP24A1 expression (198). In contrast, the 1α-hydroxylase and VDR genes are completely unmethylated (198). These results indicate that that the placenta is capable, and even "primed" for initiating significant endocrine and paracrine vitamin D response.

In mice, VDR plays a significant role in placental Ca flux. Heterozygote fetuses of VDR knockout (KO) mothers exhibit defective skeletal mineralization, increased numbers of osteoclastic cells, hypercalcemia and a five-fold increase in $1,25(OH)_2D$ (199). However, the role of vitamin D and VDR in regulation of Ca flux and fetal skeletal development may only be critical when maternal Ca intake is low enough that Ca demand cannot be met by passive absorption in the gut. This is exhibited in the VDR KO mouse; when fed a high Ca/P/lactose diet, all of the disturbed Ca phenotypes of heterozygote pups were restored to normal (199). Furthermore, heterozygote, homozygous KO, and wild type (WT) littermates of heterozygote

dams have equivalent serum Ca, P, Mg, and PTH, and neither heterozygote or KO pups exhibit alterations in placental ^{45}Ca flux, skeletal morphology or skeletal Ca and Mg content (200). However, homozygous KO pups of heterozygous dams are smaller at birth and exhibit endocrine disturbances including serum 1,25$(OH)_2$D concentrations three times higher than WT littermates (200). The lack of dramatic fetal phenotype in KO pups of heterozygous dams, combined with the rescue of fetal phenotypes from KO dams with high Ca diets has led some to propose that VDR plays a less significant role in placental Ca transfer or fetal bone mineralization in humans as well as mice (127;200). However, extrapolating data from genomic murine knockout models to human pregnancy has limitations. Data regarding placental VDR expression in humans is limited and it may be that the VDR plays a role in regulating placental Ca flux and fetal skeletal development, especially in adolescent pregnancies.

VI. Stable Ca Isotopes

Current understanding of placental Ca flux is limited by a lack of *in-vivo* human data, as extrapolation of results from animal studies must be made with caution. Dynamic measures of *in-vivo* Ca flux can be safely obtained in humans using stable Ca isotopes. In nature, Ca is comprised of six stable isotopes; ^{40}Ca is the most abundant, but five minor abundance isotopes are found at fractional abundances under 2.1% (^{42}Ca, ^{43}Ca, ^{44}Ca, ^{46}Ca, and ^{48}Ca). The ^{44}Ca isotope (found in nature at 2.06%) is commonly administered orally in human metabolic studies; ^{42}Ca (found in nature at 0.647%) and ^{46}Ca (found in nature at 0.004%) are often administered intravenously. Because these stable isotopes exist naturally and are present in the body and food supply, they pose no risk to mother or fetus at the doses administered. Two of these minor abundance isotopes (^{42}Ca and ^{44}Ca) are used in this research.

Pregnancy is characterized by an expansion of blood volume and increased bone turnover both of which impact the size of the miscible Ca pool. Thus use of an intravenous (i.v.) dose of Ca isotope is necessary in order to provide an estimate of this pool. The intravenous dose can be viewed as a proxy for 100% absorption and will give an estimate of the miscible Ca pool in each individual which will differ depending on individual variation in: body size, dietary intake, intestinal Ca absorption, endogenous fecal losses of Ca, urinary excretion of Ca, bone turnover (resorption verses deposition), and Ca transfer to the fetus. Thus, the ratio of oral**:**i.v. (^{44}Ca**:**^{42}Ca) enrichment of maternal blood post-dosing will provide a relative estimate of fractional absorption from the gut. **Figure 1.3** below demonstrates the relative sources of loss and entry into the miscible Ca pool that vary on an individual basis.

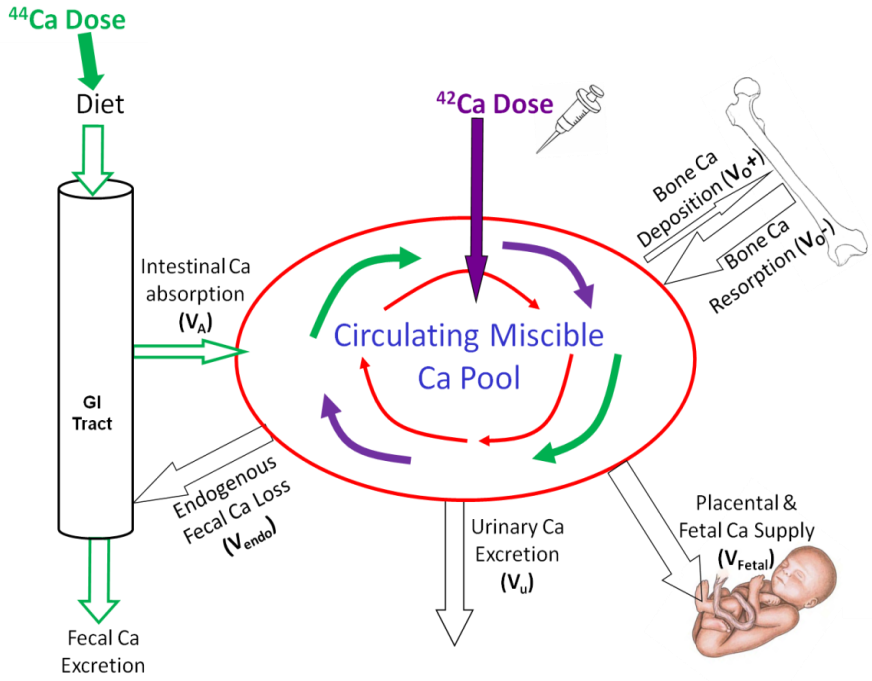

Figure 1.3 Model of Calcium Dynamics that Impact Calcium Balance During Pregnancy
Rates of entry into and excretion from the miscible Ca pool are altered during pregnancy and vary on an individual basis. Calcium enters the miscible Ca pool from absorption of dietary Ca intake (V_A) and resorption from bone (V_O-). Calcium is lost to endogenous fecal losses (V_{endo}), renal losses in the urine (V_u), bone Ca deposition (V_O+), and Ca transfer to the fetoplacental unit (V_{Fetal}). In this study ^{44}Ca was administered orally (green arrow), and then assessed in maternal circulation and neonatal circulation (cord blood). Intravenous ^{42}Ca was administered directly into the miscible Ca pool, (purple arrow). Enrichment of ^{42}Ca in maternal circulation is a function of the size of the miscible Ca pool and rates of exit from this compartment. Enrichment of ^{42}Ca and ^{44}Ca in neonatal circulation will provide insight into the degree of Ca transferred from maternal to fetal circulation.

The ratio of fetal to maternal enrichment provides a measure of the fraction of Ca administered that was partitioned to the fetus. The $^{42}Ca_{fetal}$:$^{42}Ca_{maternal}$ ratio provides a measure of the fraction of Ca administered intravenously that was transferred to the fetus; the $^{44}Ca_{fetal}$:$^{44}Ca_{maternal}$ ratio provides a measure of the fraction of dietary Ca absorbed from the gut that was then partitioned to the fetus. These measures obtained can be examined in relation to

variation in Ca intake and vitamin D status and VDR expression in the placenta. To our knowledge, such *in-vivo* data do not currently exist in humans using either radio- or stable Ca isotopes.

VII. The Teen Bone Study

This research was undertaken in the context of the "Teen Bone Study" – a study designed to investigate the impact of maternal Ca intake and vitamin D status on rates of maternal bone loss and fetal bone growth in pregnant adolescents. The study employed a longitudinal cohort design and was funded by a grant from the USDA. Inclusion criteria included: maternal age ≤ 18 years, gestational age between 12 and 30 weeks at entry into the study, carrying a singleton fetus, and otherwise healthy. Participants attended up to three study visits across pregnancy. At each visit, dietary intake, supplement usage, maternal anthropometrics, and physical activity were recorded. Maternal bone quality was monitored by calcaneous heel ultrasounds and fetal bone growth was monitored by fetal sonogram at each visit. Maternal blood was collected at the mid-gestation visit and at parturition. Cord blood and placental tissue were obtained at birth. **Figure 1.4** below represents a schematic of the study design and summarizes the main categories of data obtained from each participant.

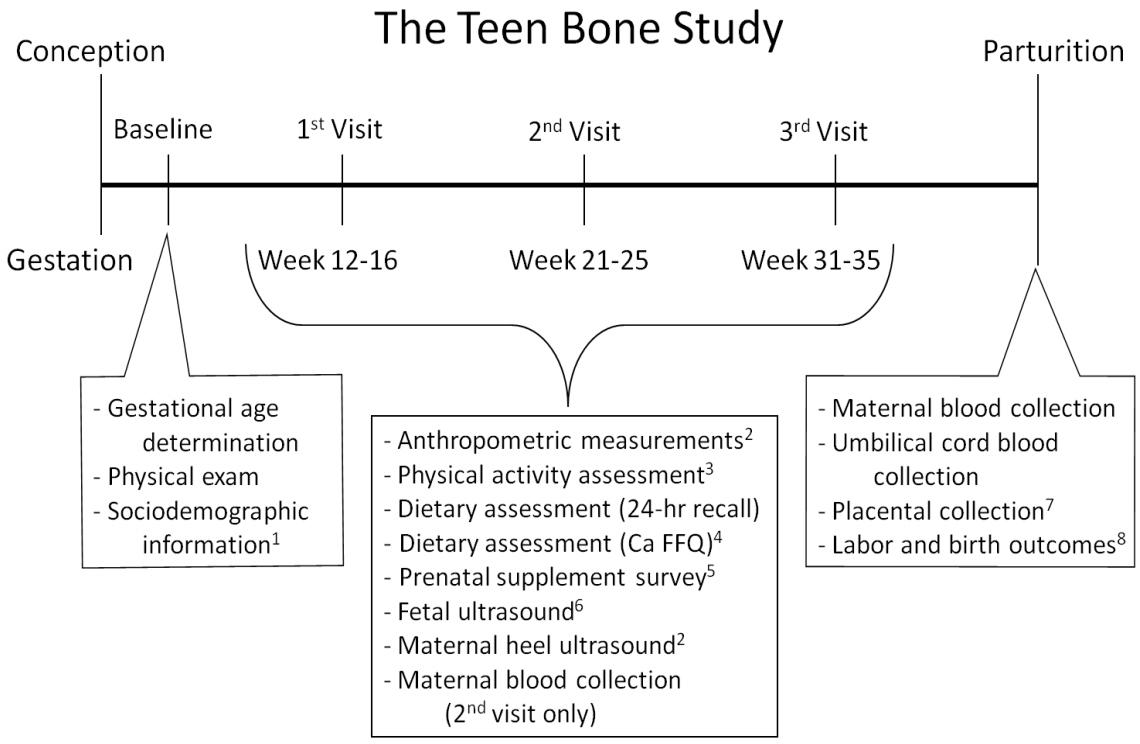

Figure 1.4 Timeline of the "Teen Bone Study"
The Teen Bone study was a longitudinal cohort study designed to characterize maternal bone loss and fetal bone growth in adolescent pregnancy, in relation to maternal Ca intake and vitamin D status. Participant data was collected using standardized, computerized data collection forms (teleforms) that are included as appendices (indicated by individual superscripts).

The study was initiated in Baltimore, MD in 2005. Thirteen participants completed the study in Baltimore before the study was moved to Rochester, NY. In 2006, study recruitment began at the Rochester Adolescent Maternity Program (RAMP), Rochester, NY. Adolescents who attended RAMP received prenatal care from the Strong Midwifery Group at a clinic located in the inner-city of Rochester. Adolescents delivered their infants with the same midwifery group at Highland Hospital, which is affiliated with the University of Rochester School of Medicine. The study was active in Rochester between December, 2006 and June, 2010 when the last participant delivered her infant. In the state of New York, pregnant adolescents (< 18 years)

are treated as emancipated minors for the duration of their pregnancy. All participants who were ≥ 15 years of age signed a study-consent form (presented in **Appendix 9**). Adolescents who were between 15 and 17 years were assigned a patient advocate. Adolescents who were between 12 and 14 years of age signed a different version of this consent that contained more age-appropriate language. For these younger participants, parental approval was also required by the University of Rochester IRB.

All data were collected on standardized computerized data collection forms (teleforms) that were organized in the following categories: maternal demographics (collected at recruitment); 24-hour dietary recall and Ca food frequency questionnaire (FFQ), supplement usage, maternal anthropometrics, fetal sonogram, and physical activity recall (all administered at each study visit); and infant data and placenta characteristics (collected at delivery). These eight teleforms are presented as **Appendices 1 – 8**. The initial study design included assessment of the following biochemical measures in all three blood samples obtained from each maternal/neonatal dyad: 25(OH)D, PTH, $1,25(OH)_2D$, leptin, estradiol, osteocalcin (OC: a biochemical marker of bone formation), N-telopeptide (NTX: a biochemical marker of bone resorption), and osteoprotegerin (OPG: a decoy receptor that facilitates osteoblast/osteoclast communication and plays a role in regulation of rates of bone turnover).

A total of 171 adolescents participated in this study. This study represents a wealth of data which can be used to investigate a multitude of research questions in addition to the original study aims. My doctoral research focuses on a sub-section of the initial aims of the grant; 1) investigations of calcitropic hormone status across pregnancy, 2) the impact of maternal Ca intake and vitamin D status on fetal bone length and length at birth, 3) the determinants of placental VDR expression, and associations with fetal bone length, and 4) in-vivo

characterization of maternal-to-fetal Ca transfer, and associations with fetal bone length. My research has a "fetal focus", and thus will not present data regarding maternal bone loss. I have also completed a manuscript detailing concentrations of OPG, OC, and NTX, and relationships between these variables and infant birth outcomes (**Appendix 11**).

Impact

The prevalence of teenage pregnancy, and the high maternal and fetal risks associated with early childbearing give this research significant public health relevance. The information obtained from this project will improve our understanding of Ca and vitamin D physiology during pregnancy, will have clinical relevance for pregnant adolescents, and will inform recommendations for adequate Ca intake in this age group to support optimal maternal and fetal bone health.

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CHAPTER 2

VITAMIN D INSUFFICIENCY IS PREVALENT AND VITAMIN D STATUS IS INVERSELY ASSOCIATED WITH PTH AND CALCITRIOL IN PREGNANT ADOLESCENTS*

*Bridget V Essley, Thomas J McNanley, Elizabeth M Cooper, Allison W McIntyre, Frank Witter, Z Leah Harris, Kimberly O O'Brien. Vitamin D Insufficiency is Prevalent and Vitamin D is Associated with PTH and Calcitriol in Pregnant Adolescents.

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ABSTRACT

Few large studies have assessed changes in calcitropic hormones and maternal 25(OH)D status across pregnancy and how this may impact neonatal hormone status. We aimed to identify determinants of 25(OH)D, PTH, and calcitriol across pregnancy in a longitudinal study of 168 pregnant adolescents (≤ 18y of age). Maternal 25(OH)D, PTH, and calcitriol were assessed at mid-gestation (~26 weeks), delivery, and in cord blood. Data were related to maternal anthropometric, dietary, and physical activity information. Insufficient 25(OH)D (≤ 20 ng/mL) was prevalent (~50%) in these adolescents and their infants; 25(OH)D was lower in African Americans versus Caucasians ($p < 0.001$). PTH increased across gestation ($p < 0.001$), and elevated PTH was detected in 40% of adolescents at delivery. Calcitriol decreased significantly by 5% across the final 13 weeks of pregnancy ($p < 0.001$), and was lower in Caucasians versus African Americans at delivery ($p < 0.040$). PTH and calcitriol did not significantly differ across the range of Ca intakes observed (257-3220 mg/day). In the group as a whole, PTH was inversely associated with 25(OH)D in maternal circulation at mid-gestation ($p = 0.023$) and at delivery ($p = 0.019$). However, when the cohort was partitioned by 25(OH)D sufficiency, this relationship was only present in those with 25(OH)D ≤ 20 ng/mL, suggestive of a threshold below which 25(OH)D impacts PTH during pregnancy. Mid-gestation 25(OH)D was also inversely associated with calcitriol at delivery ($p = 0.023$), irrespective of Ca intake. Concentrations of PTH and calcitriol in neonatal circulation were significantly lower than ($p < 0.001$), but unrelated to concentrations in maternal circulation. These findings indicate that maternal 25(OH)D status plays a role in calcitropic hormone regulation in pregnant adolescents.

INTRODUCTION

Calcitropic hormones such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, calcitriol) regulate serum calcium (Ca) concentrations and maintain whole body Ca homeostasis. Metabolic regulation of these hormones is particularly important during pregnancy to accommodate the 30 g of Ca necessary to mineralize the fetal skeleton. To help meet this physiological demand, intestinal Ca absorption increases early in pregnancy and remains elevated upwards of two-fold until term (1-3). Biochemical markers of bone resorption and bone formation increase significantly across gestation, suggesting that metabolic activity of bone is stimulated to accommodate fetal skeletal mineralization (4). While these pregnancy-associated adaptations are likely impacted by Ca intake, 25-hydroxyvitamin D (25(OH)D), PTH and $1,25(OH)_2D$, little has been published regarding determinants and interrelationships between these measures across pregnancy.

The best biomarker of vitamin D status is serum 25(OH)D, as circulating concentrations of this pro-hormone are derived from both dietary intake and endogenous dermal synthesis of vitamin D. During pregnancy, circulating 25(OH)D concentrations remain fairly constant in spite of marked increases in plasma volume (2;4). Numerous studies have documented vitamin D insufficiency ($\leq$ 20 ng/mL) during pregnancy (5), yet little is known about relationships between this pro-hormone and circulating concentrations of $1,25(OH)_2D$ and/or PTH across pregnancy, especially in racially diverse populations.

Serum $1,25(OH)_2D$ is consistently elevated during pregnancy and increases are seen as early as 8-10 weeks of gestation (3). While concentrations are typically elevated, interpretation of existing data on $1,25(OH)_2D$ concentrations in pregnant women is constrained by the limited

number of studies addressing this topic and the relatively small samples sizes (n = 10 - 40) and variety of different methodologies used to characterize status of this hormone (2-4;6-8).

In contrast to the consistent elevations typically reported for calcitriol, circulating PTH concentrations are reported to remain unchanged, decrease, or increase across gestation (4;9;10). These variable findings may be impacted by differences in 25(OH)D (11), habitual Ca intake and/or maternal age (10) between studies. Similar to existing calcitriol data, the normative data regarding PTH during pregnancy have been largely obtained from small cohorts of well-nourished, Caucasian, adult women. Few studies have examined associations among racially or ethnically diverse populations, in spite of well-described racial differences in calcitropic hormones (12).

Data regarding possible interrelationships among $1,25(OH)_2D$, PTH and 25(OH)D across pregnancy are conflicting (6;7). In non-pregnant populations, 25(OH)D has been found to be inversely associated with serum PTH, especially in the context of suboptimal 25(OH)D status (13). The recent IOM report on Ca and vitamin D requirements found "fair evidence that this relationship may exist during pregnancy" (14), but additional data are clearly needed. Variability in dietary Ca intake likely contributes to the differences observed between studies, yet few studies control for the impact of habitual Ca intake on the observations noted.

The developing fetus is solely dependent on maternal supply of 25(OH)D, and neonatal 25(OH)D concentrations are strongly correlated with maternal concentrations. In contrast, fetal $1,25(OH)_2D$ is influenced by maternal (15), placental (16), and fetal synthesis (17) of this hormone. At present the relative contributions of each source are unknown (18). Data addressing relationships between maternal and neonatal $1,25(OH)_2D$ have found that neonatal concentrations are approximately 46% lower than those observed in maternal circulation (19-21).

Similarly, neonatal PTH concentrations are approximately 26% lower than, but not correlated with, maternal PTH concentrations at birth (19).

In an attempt to address gaps in knowledge in this area, we undertook a longitudinal study to assess temporal changes and interrelationships among 25(OH)D, $1,25(OH)_2D$, PTH and Ca intake across pregnancy. This study was undertaken in a large cohort of racially and ethnically diverse pregnant adolescents; a group that may have additional Ca demands of maternal growth superimposed upon those of fetal skeletal mineralization. The impact of maternal hormonal status on neonatal calcitropic hormone status at birth was also explored.

MATERIALS AND METHODS

Participants and Study Procedures

A cohort of 171 pregnant adolescents (≤ 18 y) was recruited to participate in this prospective longitudinal study. Thirteen participants were recruited from the Maternity Center East Clinic in Baltimore, MD, and followed until delivery at Johns Hopkins Hospital (Baltimore, MD). All other study volunteers (n = 158) were recruited from the Rochester Adolescent Maternity Program (RAMP) clinic in Rochester, NY. Pregnant adolescents were eligible to participate if they were between 12 and 30 weeks gestation at entry into the study, were otherwise healthy, and were carrying a single fetus. Exclusion criteria included known medical complications such as: HIV infection, diabetes, diagnosed eating disorders, or malabsorption diseases. Informed written consent was obtained from all participants, and study procedures were approved by the Institutional Review Boards of the University of Rochester, Cornell University, and Johns Hopkins University. Maternal race, ethnicity, pre-pregnancy weight, and smoking history were self-reported. Infant race was classified as African American if the mother reported both herself and the father as African American; as Caucasian if the mother reported both herself and the father as Caucasian; or biracial if maternal and paternal race differed. Infant ethnicity was similarly classified as either: Hispanic, Non-Hispanic, or multi-ethnic.

Each adolescent was asked to attend up to three study visits across pregnancy at early, mid, and late gestation. At each study visit, maternal anthropometrics were recorded and a 24-hour dietary recall, prenatal supplement survey, and physical activity survey administered. At birth, maternal weight and infant weight and length were recorded by clinical staff using standard procedures.

Dietary and Physical Activity Assessment

Dietary recall data were obtained at each study visit by study personnel utilizing food models to help estimate portion sizes. All 24-hour recalls were analyzed by a registered dietitian using the Nutrition Data System for Research (NDSR: University of Minnesota, Minneapolis, MN, versions 2006, 2008, and 2009) in the Clinical Research Center at the University of Rochester (Rochester, NY). To assess overall physical activity, the adolescent self-reported her previous day's physical activity in 30-minute intervals using a previous day physical activity report (PDPAR) validated for students in grades 7-12 (22). A physical activity score was calculated from this survey.

Biochemical analyses

Maternal blood (10 mL) was obtained at mid-gestation (~26 weeks) and at delivery, and a 10 mL cord blood sample was obtained at delivery. Serum was separated and stored at -80^0 C until analysis. Season of each blood collection was classified as winter (November-February), spring (March-April), summer (May-August), or autumn (September-October), using similar seasonal classification groupings utilized in other studies undertaken in the northeastern United States (23).

In each serum sample collected, serum total Ca concentration was measured using a Modular (P) Chemistry Automated System (Roche Diagnostics, Indianapolis, IN) and 25(OH)D was measured using the Diasorin RIA (Diasorin Inc, Stillwater, MN) by Quest Laboratories. This laboratory participates in the vitamin D External Quality Assessment Scheme (DEQAS). Vitamin D deficiency was defined as 25(OH)D < 10 ng/mL and vitamin D insufficiency as 25(OH)D ≤ 20 ng/mL (14). After a high prevalence of vitamin D insufficiency was observed in the first 45 participants, all subsequent adolescents found to be vitamin D insufficient at mid-

gestation were provided a bottle of daily supplements containing 400 IU of vitamin D_3 at their next prenatal visit, to take daily over the remainder of gestation. Calcitriol ($1,25(OH)_2D$) was analyzed at Boston University in the lab of Dr. Michael Holick (Boston, MA) using an in-house thymus receptor binding assay as previously described (24). Intact PTH (iPTH) and leptin were analyzed using commercially available ELISAs (DSL Laboratories, Webster, TX and Millipore, Billerica, MA, respectively). Elevated PTH was defined if PTH concentrations were ≥ 46 pg/mL (11).

Statistical Analyses

Analyses were performed using SAS 9.2 and JMP 8.0 (SAS Institute Inc, Cary, NC). Results are reported as the mean ± standard deviation (SD) unless otherwise stated. Paired t-tests or non-parametric tests were used to assess changes in hormones across gestation within subjects. Independent t-tests or ANOVA were used to determine if normally distributed variables differed by either race, season, or tertiles of Ca intake; the Wilcoxon Rank sum test was utilized for nonparametric data. Differences in 25(OH)D, PTH, and $1,25(OH)_2D$ between mid-gestation, delivery, and cord blood within subjects were tested, controlling for participant ID as a fixed effect and utilizing a Tukey correction. Simple linear regression was used to assess relationships among 25(OH)D, PTH, and $1,25(OH)_2D$ at each time point. Simple and multiple linear regressions were used to model statistical predictors of circulating 25(OH)D, PTH, and $1,25(OH)_2D$ in the adolescent and neonate. Variables were tested for normality using the Shapiro Wilks test. Non-normally distributed variables were log transformed as necessary to ensure normality of the residuals. P values < 0.05 were considered significant, and P values between 0.05 and 0.10 were identified as trends of interest.

RESULTS

Subject Characteristics

Characteristics of the study population as a whole, and when stratified by race are presented below in **Table 2.1**.

Table 2.1.

Characteristics of Pregnant Adolescents and their Neonates at Birth[1]

Subject Characteristics	All Subjects	African American Adolescents	Caucasian Adolescents
Total Recruitment	168	66.1% (111)	33.9% (57)
Age at Enrollment (years)	17.1 ± 1.1 (167)	17.1 ± 1.2 (110)	17.2 ± 0.9 (57)
Hispanic Non-Hispanic	24.1 % (39) 75.9% (123)	6.6 % (7)[2] 93.4% (99)	57.1 % (32) 42.9 % (24)
Ca Intake (mg/day)	913 ± 414 (162)	886 ± 421 (106)	963 ± 400 (56)
Vitamin D Intake (µg/d)	5.4 ± 3.4 (162)	5.3 ± 3.4 (106)	5.7 ± 3.3 (56)
Pre-Pregnancy BMI (kg/m^2)	24.7 ± 5.3 (163)	24.4 ± 5.1 (106)	25.2 ± 5.7 (57)
Parity ≥ 1	9.2% (167)	9.1% (110)	9.3% (57)
Gestational Age at Enrollment (weeks)	22.1 ± 5.7 (165)	22.6 ± 5.8 (110)	21.1 ± 5.5 (57)
Gestational Age at Delivery (weeks)	39.2 ± 2.9 (162)	39.2 ± 2.8 (107)	39.2 ± 3.1 (55)
Birth Weight (g)	3236 ± 592 (159)	3207 ± 607 (105)	3293 ± 561 (54)
Preterm	8.6% (162)	8.4% (107)	9.1% (55)
Birth Weight > 4500	8.8% (159)	9.5% (105)	7.4% (54)
Birth Weight < 2500	6.29% (159)	5.71 (105)	7.41% (54)

[1]Data are reported as the Mean ± SD, or percentage, with the sample size in parentheses.
[2]Significantly different than Caucasians: $p<0.0001$.

Biochemical samples were not obtained from two Baltimore participants and one Rochester participant. Because vitamin D status in these adolescents was unknown, they were excluded from all analyses making the final sample size 168. Data regarding circulating

concentrations of osteoprotegerin and markers of bone turnover in a subset of this cohort have been previously reported (25). Two of the 168 participants self-identified their race as American Indian. None of the race-specific analyses differed if data were analyzed with these two adolescents combined with either the African American or Caucasian cohort. In order to collapse maternal race into a bivariate category, data from the two American Indian adolescents were included within the African American cohort. Maternal age at enrollment into the study ranged from 13.6 to 18.7 years. Gestational weight gain was 16.8 ± 8.0 kg on average (ranging from -2.3 to 43.1 kg). The mid-gestation blood collection was obtained at 26.3 ± 3.6 weeks gestation, on average. Length of gestation ranged from 20.7 to 43.0 weeks, and birth weight ranged from 1,054 to 4,705 g. Infant sex did not differ by race, and 53.1% of infants were male. A significantly higher percentage of Caucasian participants self-identified as Hispanic (57.1%) compared to African American (6.6%) participants; $p<0.0001$. Other than ethnicity, there were no other racial differences in maternal or infant characteristics including; length of gestation, birth weight, or rate of preterm, low birth weight (LBW: <2500g), or large for gestational age infants (LGA: >4500g) (**Table 2.1**).

Dietary Intake

Daily dietary caloric, vitamin D and Ca intakes recorded at different study visits across gestation did not significantly differ within subjects. Thus, the mean intake of all study visits for each adolescent was used. On average, adolescents consumed 2,320 ± 740 calories, of which of 52%, 14% and 34% were obtained from carbohydrates, protein, and fat, respectively. Calcium intake ranged from 257 to 3220 mg/day. Only 29% of adolescents met the EAR for Ca (1100 mg/day) and only 16% met the RDA (1300 mg/day) (14). Vitamin D and Ca intake were both positively associated with the amount of calories consumed ($p<0.0001$, $n = 162$), and were also

highly correlated with each other ($p<0.0001$, $R^2 = 0.462$, n = 162). Dietary caloric, vitamin D, and Ca intake did not significantly differ as a function of maternal race. Supplemental vitamin D was provided to 25(OH)D insufficient adolescents at 30.3 ± 3.5 weeks, on average. Self-reported data from the prenatal supplement survey indicated that only 8.9% of teens consumed the provided vitamin D_3 supplement on a daily basis.

Vitamin D Supplementation

Supplemental vitamin D_3 (400 IU/day) was offered to teens whose mid-gestation 25(OH)D concentrations were ≤ 20 ng/mL, who had enrolled in the study after the first 45 participants had enrolled. Adolescents who received supplements (n = 49) were more likely to be African American (79.6% vs. 61.0%, p = 0.016), and had a higher pre-pregnancy BMI (25.9 ± 5.7 vs. 24.2 ± 5.1 kg/m^2, p = 0.050), but were otherwise similar in characteristics to the unsupplemented group. Supplements were provided at 30.3 ± 3.5 weeks gestation. Those who were vitamin D insufficient but did not receive supplements either joined the study before the supplements were provided (n = 19), failed to return to the clinic to receive the supplements (n = 15), or refused to accept them (n = 4). Self-reported data indicated that only 26.4% of adolescents consumed these supplements daily.

Longitudinal Changes and Predictors of 25(OH)D, PTH, and 1,25(OH)$_2$D

The biochemical measures of calcitropic hormone status in these adolescents and their neonates are shown for the cohort as a whole, and when separated by maternal race in **Table 2.2**. Approximately 50% of adolescents were vitamin D insufficient (25(OH)D ≤ 20 ng/mL), with African American participants having a significantly higher prevalence of insufficiency compared to Caucasians at mid-gestation (p = 0.002) (**Table 2.2**).

Table 2.2.

Serum Vitamin D and PTH concentrations across Gestation in Pregnant Adolescents and in their Neonates at Birth[1]

	All Subjects	African American	Caucasian
25(OH)D (ng/mL)			
Mid-Gestation [€] % ≤ 20 (n)	22.1 ± 10.2[a] 52.4% (166)	20.5 ± 9.9[a] 60.6% (110)	22.5 ± 9.8[a] 35.7% (56)
Delivery [#] % ≤ 20 (n)	21.5 ± 11.6 [a] 50.3% (137)	19.8 ± 10.1 [a] 54.0% (87)	24.4 ± 13.4 [a] 42.0% (50)
Cord Blood [€] % ≤ 20 (n)	20.8 ± 10.2 [a] 52.8% ≤ 20 (123)	16.6 ± 9.4 [a] 61.5% (50)	24.6 ± 10.6 [a] 37.8% (45)
PTH (pg/mL)			
Mid-Gestation [Δ] % > 46 (n)	28.7 ± 16.0 [a] 12.1% (91)	32.2 ± 18.2 [a] 19.6% (56)	23.0 ± 9.4 [a] 0% (35)
Delivery % > 46 (n)	45.1 ± 29.4 [b] 40.0% (80)	47.1 ± 33.2 [b] 43.1% (51)	41.5 ± 21.0[b] 34.5% (29)
Cord Blood % > 46 (n, % detectable)	19.0 ± 12.1 [c] 0% (33, 37%)	19.3 ± 12.2 [c] 0% (22, 39%)	18.2 ± 12.5 [c] 0% (11, 33%)
$1,25(OH)_2D$ (pg/mL)			
Mid-Gestation (n)	117 ± 30 [a] (100)	118 ± 30 [a] (63)	115 ± 31 [a] (37)
Delivery [#] (n)	106 ± 31 [b] (96)	111 ± 31 [b] (63)	97 ± 29 [b] (33)
Cord Blood (n)	48 ± 19 [c] (74)	48 ± 17 [c] (49)	46 ± 23 [c] (n = 25)

[1]Data are reported as the Mean ± SD, or percentage, with the sample size in parentheses.
Mid-Gestation = 26.3 ± 3.6 weeks; Delivery = 39.2 ± 2.9 weeks.
Races differ at the given time-point: [#]: $p < 0.05$; [Δ]: $p < 0.01$; [€]: $p < 0.001$
Different letter superscripts statistically differ within subjects, between Mid-Gestation, Delivery, and Cord Blood values; $p < 0.05$.

Trends in 25(OH)D, PTH, and 1,25(OH) $_2$D from mid-gestation to delivery as a function of race are depicted below in **Figure 2.1**.

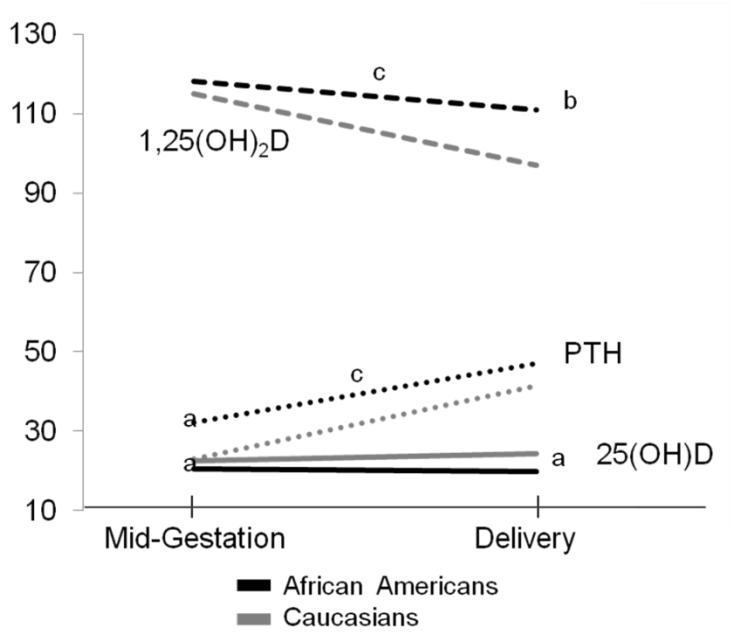

Figure 2.1 Temporal Trends in 25(OH)D, 1,25(OH)$_2$D, and PTH Concentrations as a Function of Race in Pregnant Adolescents
Temporal trends in 25(OH)D (ng/mL), PTH (pg/mL), and 1,25(OH)$_2$D (pg/mL) from mid-gestation (23.3 ± 3.6 weeks) to delivery (39.2 ± 2.9 weeks). Caucasians had higher concentrations of 25(OH)D at both mid-gestation and delivery (a: $p < 0.0001$). Serum PTH was higher in African American adolescents at mid-gestation (a: $p < 0.0001$), and increased from mid-gestation to delivery in both races (c: $p < 0.0001$). Serum 1,25(OH)$_2$D decreased from mid-gestation to delivery in both races (c: $p < 0.0001$), and was higher in African Americans at delivery (b: $p < 0.040$). The mean change in all three measures from mid-gestation to delivery did not significantly differ between the African American and Caucasian adolescents.

Maternal race and season of blood collection were both associated with maternal 25(OH)D at mid-gestation and at delivery. Caucasians had higher 25(OH)D at both time-points (mid-gestation: $p < 0.0005$, delivery: $p = 0.034$), and 25(OH)D was higher in the summer versus the winter ($p < 0.040$). Physical activity score at mid-gestation was positively associated with

maternal 25(OH)D status at mid-gestation ($p = 0.005$, $R^2 = 0.049$), and delivery ($p = 0.014$, $R^2 = 0.045$). Adolescents consuming Ca intakes within the third tertile (> 1060 mg/d) had significantly higher 25(OH)D at mid-gestation ($p = 0.034$) and at delivery ($p = 0.006$). Vitamin D intake during pregnancy was positively associated with 25(OH)D status at delivery only ($p = 0.004$, $R^2 = 0.062$). Maternal 25(OH)D concentrations were inversely related to maternal weight at mid-gestation ($p = 0.034$, $R^2 = 0.027$), but not at delivery. Maternal age, BMI, weight gain, and leptin concentrations were not significantly associated with 25(OH)D in maternal circulation at either time point. Teens who received vitamin D supplements continued to exhibit significantly lower 25(OH)D concentrations at delivery (17.9 ± 7.3 ng/mL vs. 23.1 ± 13.0 ng/mL, $p = 0.001$) and a higher prevalence of vitamin D insufficiency (63.3% vs. 44.4%, $p = 0.03$) at delivery compared to unsupplemented teens.

In the cohort as a whole, concentrations of 25(OH)D did not significantly differ within subjects between the mid-gestation and delivery measures, and the average change over the 12.9 ± 4.4 weeks study interval was -0.5 ± 11.7 ng/mL. The change in 25(OH)D concentrations in those who received supplements (3.2 ng/mL, $n = 45$) was significantly higher than the change observed in unsupplemented adolescents (-2.3 ng/mL, $n = 90$, $p = 0.0002$). Among non-supplemented adolescents only, the change in 25(OH)D from mid-gestation to delivery was larger in those who delivered in the summer and autumn compared to those who delivered in the spring ($p = 0.020$, $n = 90$).

PTH at mid-gestation was significantly lower in Caucasians ($p = 0.012$), but this difference was not evident at delivery. PTH was not associated with gynecological age or Ca intake and did not differ between teens who received and did not receive vitamin D supplementation at any time point measured. At mid-gestation, 12.1% of adolescents exhibited

elevated PTH (≥ 46 pg/mL) and PTH concentrations were significantly higher in African American adolescents compared to Caucasians, by 9.2 pg/mL (**Table 2.2**). Over the average ~13 weeks that elapsed between the mid-gestation and delivery blood draw, PTH increased significantly by 16.3 ± 26.6 pg/mL, ($p<0.0001$) in the cohort as a whole. This increase was significant in both the Caucasian and African American cohorts ($p<0.001$), and the magnitude of increase did not differ by race (**Figure 2.1**), Ca or vitamin D intake, receipt of vitamin D supplementation, or season of blood draw. By delivery, the prevalence of elevated PTH had more than doubled, reaching 40.0%.

Caucasian teens had significantly lower $1,25(OH)_2D$ than African American adolescents at delivery ($p = 0.037$) (**Table 2.2**). Calcitriol at delivery was significantly higher in adolescents who received vitamin D supplements compared to those who did not (115.8 ± 32.3 vs. 100.9 ± 28.6 pg/mL, $p = 0.036$). Calcitriol at mid-gestation was positively associated with pre-pregnancy BMI ($p = 0.028$, $R^2 = 0.022$) and maternal weight at the time of blood collection ($p = 0.010$, $R^2 = 0.065$). Calcitriol at mid-gestation was also positively associated with estradiol ($p = 0.010$, $R^2 = 0.065$) and with gestational age over the ~17 week interval across which this blood collection was obtained ($p < 0.001$, $R^2 = 0.108$). When both estradiol and gestational age were entered as covariates into a model predicting $1,25(OH)_2D$ at mid-gestation, only gestational age remained a significant predictor of $1,25(OH)_2D$. Calcitriol was unrelated to maternal age or Ca or vitamin D intake at any time point measured.

Calcitriol decreased on average by 10.9 ± 34.9 pg/mL (5.0 ± 32%) from mid-gestation to delivery. The decrease was significant ($p = 0.005$) in the cohort as a whole, and within each race individually (**Table 2.2**). While $1,25(OH)_2D$ was significantly higher in African American adolescents at delivery, the observed decrease in calcitriol did not significantly differ by race

(**Figure 2.1**), or as a function of average Ca or vitamin D intake, season of blood collection, or receipt of vitamin D supplements. The decrease in $1,25(OH)_2D$ was larger in adolescents with 25(OH)D > 20 ng/mL at delivery compared to those with insufficient 25(OH)D ($p = 0.033$). The analogous non-significant trend also existed at mid-gestation between those who were 25(OH)D sufficient vs. insufficient ($p = 0.057$).

Relationships among 25(OH)D, PTH, and $1,25(OH)_2D$ in Maternal Circulation

Possible associations between maternal 25(OH)D status and calcitropic hormone concentrations were explored. A significant increase was observed across pregnancy in the prevalence of elevated PTH in adolescents who were 25(OH)D sufficient (**Figure 2.2**).

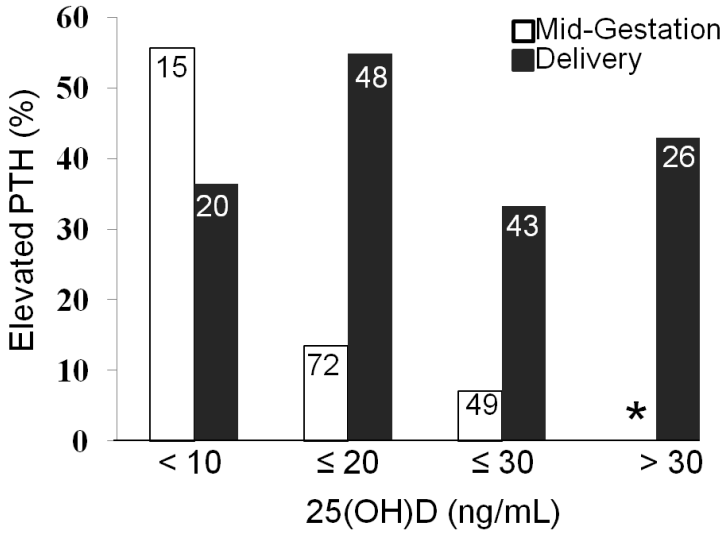

Figure 2.2 Prevalence of Elevated PTH by Categories of 25(OH)D at Mid-Gestation and at Delivery
The prevalence of elevated PTH (≥ 46 pg/mL) increased from mid-gestation (23.3 ± 3.6 weeks) to term (39.2 ± 2.9 weeks), and was detected in adolescents with replete 25(OH)D at delivery. The number of adolescents in each 25(OH)D category are indicated within each column. At mid-gestation, there were 30 adolescents with 25(OH)D > 30ng/mL, none of these teens exhibited elevated PTH (indicated by the asterisk).

At mid-gestation, 9.9% of adolescents with suboptimal 25(OH)D exhibited elevated PTH, and 2.2% of adolescents with 25(OH)D > 20 ng/mL exhibited elevated PTH. These percentages increased to 22.5% and 17.5%, respectively at delivery (**Figure 2.2**). At mid-gestation and at delivery, PTH was inversely associated with 25(OH)D in maternal circulation (mid-gestation: $p = 0.023$, $R^2 = 0.057$, $n = 91$, and delivery: $p = 0.003$, $R^2 = 0.068$, $n = 80$) (**Figure 2.3a**). When analyzed separately by race, the negative relationship between 25(OH)D and PTH was significant only among African American adolescents, both at mid-gestation ($p = 0.047$, $R^2 = 0.071$, $n = 56$ vs. $p = 0.340$, $n = 35$) and at delivery ($p = 0.008$, $R^2 = 0.133$, $n = 51$ vs. $p = 0.573$, $n = 29$). However, this racial dichotomy appeared to be driven by vitamin D insufficiency; as the negative relationship between 25(OH)D and PTH was detected in adolescents of both races who exhibited mid-gestation 25(OH)D insufficiency, but not among those with 25(OH)D > 20 ng/mL (mid-gestation: $p = 0.0243$, $R^2 = 0.110$, $n = 46$ vs. $p = 0.625$, $n = 45$, and delivery: $p = 0.036$, $R^2 = 0.106$, $n = 42$ vs. $p = 0.382$, $n = 38$). In contrast, the relationship between 25(OH)D and PTH did not differ when analyzed separately in adolescents above and below the median Ca intake (860 mg/d), nor did it differ when assessed as a function of maternal calcitriol concentration above and below the mean observed at mid-gestation (117 pg/mL) or delivery (106 pg/mL) nor was it impacted by maternal receipt of vitamin D supplements.

In addition to the association noted between 25(OH)D and PTH, maternal concentrations of 25(OH)D at mid-gestation (26.3 ± 3.6 weeks) were also inversely associated with $1,25(OH)_2D$ concentrations at delivery ($p = 0.032$, $r = -0.221$, $n = 95$) (**Figure 2.3b**). This relationship was not impacted by race or Ca intake and remained significant when examined separately among those with 25(OH)D above and below 20 ng/mL, or separately in either the African American or Caucasian cohort. Paradoxically, serum PTH was not related to $1,25(OH)_2D$ at any time point.

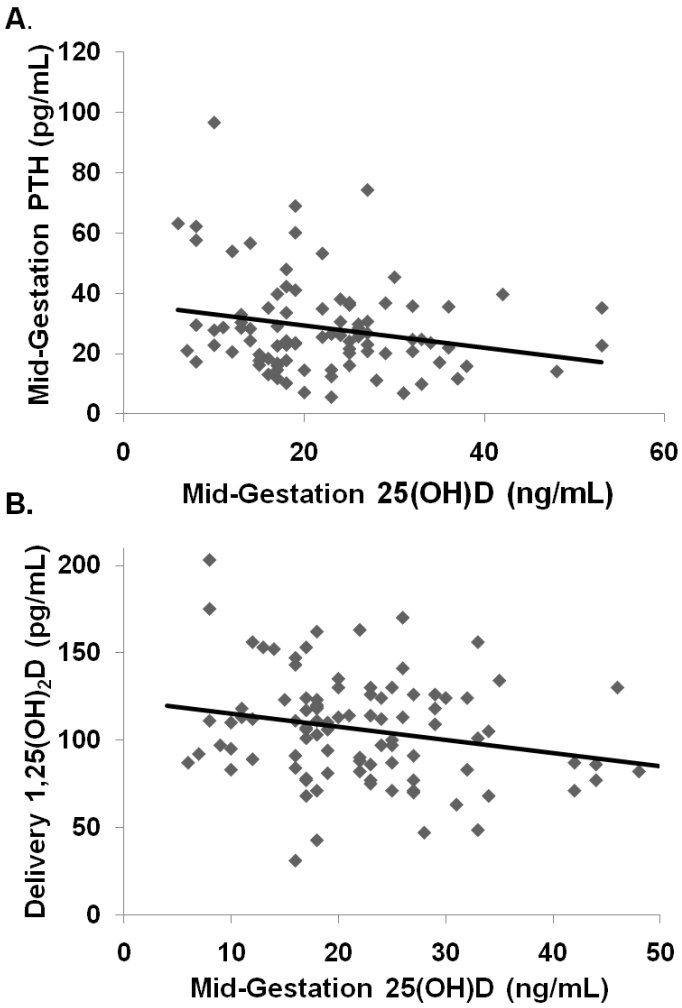

Figure 2.3 Relationships Between Maternal 25(OH)D at Mid-Gestation, PTH at Mid-Gestation, and 1,25(OH)$_2$D at Delivery in Pregnant Adolescents
A: At mid-gestation (23.3 ± 3.6 weeks) the Ln(PTH, pg/mL) was inversely associated with the Ln(25(OH)D, ng/mL): p = 0.023, n = 91, R^2 = 0.057. The untransformed relationship is presented for ease of interpretation. This relationship was maintained at delivery (data not shown: p = 0.019, n = 80, R^2 = 0.068). B: Mid-gestation 25(OH)D concentrations were inversely associated with 1,25(OH)$_2$D concentrations at delivery: p = 0.032, n = 95, R^2 = 0.049.

Predictors of Hormonal Status of the Neonate at Birth and Relationships with Maternal Status

At birth, neonates born to Caucasian mothers had significantly higher cord 25(OH)D concentrations than those born to African American mothers (p = 0.001). In the cohort as a

whole, 16.8% of neonates were vitamin D deficient (25(OH)D < 10 ng/mL), and 52.8% of neonates were vitamin D insufficient (25(OH)D ≤ 20 ng/mL). Vitamin D deficiency and insufficiency were more prevalent in neonates born to African American adolescents than among those born to Caucasian teens ($p = 0.007$). Neonatal 25(OH)D was highly correlated with ($p<0.0001$), and did not significantly differ from maternal concentrations of 25(OH)D at mid-gestation or at delivery. Similar to maternal 25(OH)D, cord 25(OH)D concentrations were positively related to maternal physical activity score at mid-gestation ($p = 0.020$, $R^2 = 0.045$), maternal vitamin D intake ($p = 0.015$, $R^2 = 0.049$), and maternal Ca intake ($p = 0.002$, $R^2 = 0.076$).

PTH was undetectable (< 10 pg/mL) in 63.3% of cord blood samples assessed and no elevated values (≥ 46 pg/mL) were observed in samples that had detectable PTH. Neonates with detectable concentrations of PTH in cord blood did not differ from those with undetectable values in terms of race, gestational age, birth weight, maternal Ca or vitamin D intake, or maternal 25(OH)D, PTH, or $1,25(OH)_2D$ concentrations. Cord blood PTH was significantly lower than ($p<0.001$), but unrelated to maternal concentrations at mid-gestation and delivery. Neonatal PTH concentrations did not differ by race, season of delivery, or maternal Ca or vitamin D intake.

Calcitriol measures were obtained in 100 neonates at delivery. Neonatal serum $1,25(OH)_2D$ averaged 58.5 ± 34.1 pg/mL and was 49.3% lower than maternal concentrations at delivery ($p <0.0001$). Neonatal concentrations remained significantly lower than maternal concentrations when examined within each racial cohort individually ($p <0.0001$). While significantly lower, cord blood concentrations of $1,25(OH)_2D$ were unrelated to maternal concentrations at mid-gestation or at delivery. Neonatal $1,25(OH)_2D$ concentrations were

negatively correlated with gestational age at delivery (35.1 – 41.9 weeks) across the 6.8 week range observed in this cohort ($p = 0.035$, $R^2 = 0.061$). No other study variables were significantly associated with neonatal $1,25(OH)_2D$ concentrations.

Of the neonates with detectable PTH in cord blood (n = 33), no significant relationships between PTH and either maternal or neonatal 25(OH)D or $1,25(OH)_2D$ were evident. Neonatal 25(OH)D was inversely associated with maternal concentrations of PTH both at mid-gestation ($p = 0.0005$, $R^2 = 0.143$, n = 82) and at delivery ($p = 0.004$, $R^2 = 0.104$, n = 76).

DISCUSSION

Vitamin D insufficiency was prevalent in this group of racially diverse pregnant adolescents. Of note, PTH concentrations increased significantly across gestation in both races, and were inversely related to 25(OH)D at both mid-gestation and at delivery. Maternal 25(OH)D at mid-gestation was also significantly inversely related to 1,25$(OH)_2$D at delivery. These relationships suggest that 25(OH)D status may have an impact on calcitropic hormone regulation during pregnancy.

The high prevalence of vitamin D insufficiency observed (~50%) was similar to other reports among pregnant women (26) and African American adolescents (27) in the northeastern United States. The expected racial and seasonal differences in 25(OH)D were observed in these adolescents. Both dietary intake of vitamin D and mid-gestation activity score (likely a proxy for sun exposure) were positively associated with maternal 25(OH)D concentrations, indicating that both intake and endogenous production of vitamin D contributed to circulating 25(OH)D in this population as a whole. However, the change in 25(OH)D status only differed by season in adolescents who did not receive vitamin D supplements, indicating that provision of daily 400 IU of vitamin D_3 blunted the effect of season on 25(OH)D in those with suboptimal concentrations. However, although supplemented teens exhibited a significantly greater increase in 25(OH)D over the final 13 weeks of pregnancy, they still exhibited a significantly higher prevalence of insufficiency at delivery compared to those who did not receive supplements. We are unable to assess whether the amount of total vitamin D provided from diet and supplements was inadequate, or if compliance with the supplements provided was poor.

Of particular note, serum PTH concentrations and the prevalence of elevated PTH in this group increased from mid-gestation to delivery, an effect that was independent of race and

vitamin D supplementation. Few other studies have reported an increase in PTH across gestation (4;9) or documented elevated PTH concentrations during pregnancy (11). Circulating PTH may increase during pregnancy to stimulate renal Ca conservation in women with suboptimal Ca intake (28), but increases in this hormone were independent of dietary Ca intake in these adolescents. It is possible that the unique Ca challenges, coupled with the suboptimal 25(OH)D status in this pediatric population may be partially responsible for our findings of increasing and elevated PTH that have not been reported in other published data from well-nourished adults. There are limitations to the assessment of PTH status from a single serum sample at mid-gestation, and at delivery. Serum used for PTH analyses must be frozen quickly after collection as PTH degrades quickly. However, increased degradation would only have diluted our ability to detect elevated intact PTH in this population. Parathyroid hormone does exhibit diurnal variation in its secretion pattern, and we did regulate the time of blood collection. However, PTH secretion peaks between 1:30 and 4:10 am in pre-menopausal non-pregnant women (29) and we obtained all mid-gestation blood samples at prenatal care appointments. Blood collections obtained at delivery were obtained at all times of day, when the adolescent was admitted for delivery of her infant. However, the amplitude of PTH secretion over the course of 24 hours is only 3.8 pg/mL (29), and thus variation in time of collection was unlikely to have inflated the prevalence of elevated PTH detected either at mid-gestation or at delivery.

Although PTH was not associated with dietary Ca intake, PTH was significantly inversely associated with maternal 25(OH)D at both time-points. This inverse relationship has been documented in other studies conducted in non-pregnant adults and adolescents (30;31). In adults, some studies have noted an inverse relationship between 25(OH)D and PTH until a minimum threshold level of 25(OH)D is achieved (31), but the inflection point at which this

association is evident remains unclear (14). In our adolescents, there did appear to be a threshold-type relationship, in that PTH was inversely related to 25(OH)D concentrations in adolescents of both races when 25(OH)D concentrations were ≤ 20 ng/mL. It is possible that the negative relationship between 25(OH)D and PTH in African Americans, but not in Caucasians, was driven by the lower 25(OH)D status of the African American cohort given that this relationship was evident in all vitamin D insufficient teens, irrespective of race. A similar inverse relationship between 25(OH)D and PTH has been detected in non-pregnant adolescents consuming Ca intakes similar to our cohort (30). In that study it was postulated that PTH secretion was increased in the context of insufficient 25(OH)D to accommodate the demands of growth (30). A similar mechanism may be present among our vitamin D insufficient teens in order to meet the combined Ca demands of both maternal and fetal growth.

This is the largest longitudinal study to asses $1,25(OH)_2D$ at multiple time points across pregnancy in an adolescent cohort. The $1,25(OH)_2D$ concentrations we observed are lower than reported in a group of 10 pregnant Nigerian adolescents assessed during the third trimester of pregnancy (137 ± 31 pg/mL) and at term (130 ± 38 pg/mL) (19), and are also lower than reported among a cohort of 20 Gambian women at 20 weeks gestation (143 ± 48 pg/mL) (32). However, the calcitriol concentrations we reported are higher than other data from healthy pregnant adult women (50 ± 9 pg/mL at 33 weeks gestation, n = 26 and 82 ± 31 pg/mL at 34-36 weeks gestation, n = 14) (3;7). Because no standard reference materials exist for $1,25(OH)_2D$, it is difficult to interpret absolute values between studies.

We noted a positive relationship between maternal $1,25(OH)_2D$ concentration at mid-gestation and the gestational age of the blood collection, across the 16.1 week window over which the mid-gestation measure was obtained. This increase over mid-gestation is consistent

with previous studies (3). In our adolescent cohort, $1,25(OH)_2D$ concentrations decreased significantly by 5% over the last trimester of pregnancy. This differs from some reports of no change in $1,25(OH)_2D$ across the last trimester of pregnancy (2;33), but is consistent with other studies that have documented a trend for a decrease in this hormone late in gestation, ranging from 5.1 – 16.7% (8;19). Because the average $1,25(OH)_2D$ concentration we observed at mid-gestation was relatively high, even with the observed 5% decrease, average delivery concentrations remained over 100 pg/mL. While the observed decrease did not significantly differ between the races, $1,25(OH)_2D$ concentrations in Caucasian adolescents were significantly lower than observed in the African American adolescents at delivery. Pregnant African American women have been shown to have significantly higher $1,25(OH)_2D_3$ and tend to have higher total $1,25(OH)_2D$ than Caucasian counterparts at term (34). Similarly, higher $1,25(OH)_2D$ concentrations have been reported among African American vs. Caucasian non-pregnant adolescent females (35). The physiological relevance of the observed 13.7 pg/mL difference in $1,25(OH)_2D$ between our African American and Caucasian adolescents is unknown. We did not measure vitamin D binding protein (DBP) in this cohort, and are thus unable to assess the degree to which the observed differences in total $1,25(OH)_2D$ are related to concentrations of free $1,25(OH)_2D$. In general, pregnancy has been associated with increases in DBP, total, and free $1,25(OH)_2D$ concentrations (36;37). Because it is the free form that is hormonally active and crosses the placenta (36), future research on changes in bound and free $1,25(OH)_2D$ across pregnancy are warranted.

A greater decrease in $1,25(OH)_2D$ was observed among teens with sufficient 25(OH)D at delivery which is consistent with the inverse relationship observed between 25(OH)D at mid-gestation and $1,25(OH)_2D$ at term. This relationship may be a potential source of the observed

racial difference in $1,25(OH)_2D$ detected at delivery, as the African Americans had lower 25(OH)D concentrations than the Caucasians at mid-gestation. The mechanisms responsible for this observation are not known. Prior work in rats fed a vitamin D deficient diet found D deficiency to result in a time-delayed 30-fold increase in the activity of the 1α-hydroxylase enzyme in renal mitochondria (38). Thus, it is possible that the chronic suboptimal 25(OH)D status of our population impacted 1α-hydroxylase activity and renal calcitriol secretion. It has also been postulated that high serum $1,25(OH)_2D$ during pregnancy may suppress 25(OH)D production via $1,25(OH)_2D$-mediated increased transcription of the 24-hydroxylase gene (39). A recent meta-analyses designed to address this hypothesis during pregnancy found no relationship between 25(OH)D and $1,25(OH)_2D$ (39), but this study contained few longitudinal data, limiting the ability to detect relationships between 25(OH)D and $1,25(OH)_2D$ that may be time-delayed. In a recent study by Hollis et al., a positive relationship was noted between 25(OH)D and $1,25(OH)_2D$, indicating that $1,25(OH)_2D$ concentrations increased as 25(OH)D increased until a threshold of 40 ng/mL was achieved (40). In that study (mean age = 27 y), 25(OH)D was increasing as pregnancy progressed due to the vitamin D supplementation all women received. It is possible that different physiological relationships regulate $1,25(OH)_2D$ production when 25(OH)D is suboptimal and remains low as pregnancy progresses. Because our study was observational, we are unable to establish the causal direction of the observed relationships between 25(OH)D and $1,25(OH)_2D$ or between 25(OH)D and PTH.

It is noteworthy that serum $1,25(OH)_2D$ concentrations were not significantly impacted by habitual Ca intake across the observed 257 - 3220 mg/day range in dietary intake. Calcitriol concentrations were also not significantly related to PTH concentrations at any time-point studied. Future research is needed to characterize the relative contribution of extra-renal tissues,

such as the placenta, to circulating $1,25(OH)_2D$. Additionally, the role of other potential regulators of the 1α-hydroxylase enzyme during pregnancy (such as PTHrP (28) and FGF23 (41)) should be examined in order to more fully explore both the regulation and role of $1,25(OH)_2D$ concentrations across pregnancy.

As expected, there were no significant differences between maternal and neonatal 25(OH)D concentrations. Neonates born to African American adolescents had lower concentrations than neonates born to Caucasian adolescents, as has been previously reported (11;34;42). While the fetus is solely dependent on maternal supply of 25(OH), it is capable of endogenous PTH and $1,25(OH)_2D$ synthesis (43;44). We observed no associations between maternal and neonatal concentrations of PTH or $1,25(OH)_2D$. Infant calcitriol values were significantly lower than maternal values, as has been reported in other smaller studies (n = 10-25 dyads) of this relationship (21;42;45). In contrast to the associations noted in the pregnant adolescents, neonatal $1,25(OH)_2D$ and PTH concentrations were not associated with neonatal 25(OH)D concentrations and did not significantly differ as a function of race.

We have demonstrated that PTH increases across gestation in pregnant adolescents, and is impacted by systemic 25(OH)D concentrations. A negative relationship between 25(OH)D at mid-gestation and $1,25(OH)_2D$ at delivery was also found. These data suggest that low 25(OH)D may impact calcitriol or PTH synthesis or conversely that calcitriol and PTH concentrations may impact metabolism of 25(OH)D. These data in this group of pregnant adolescents add to our understanding of the calcitropic hormone responses to pregnancy, and speaks to the importance of maintaining optimal vitamin D status across pregnancy. Further work addressing the impact of these hormones on maternal and fetal bone turnover is warranted.

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CHAPTER 3

MATERNAL VITAMIN D STATUS AND CALCIUM INTAKE INTERACT TO IMPACT FETAL SKELETAL GROWTH IN PREGNANT ADOLESCENTS*

*Bridget V Essley, Thomas J McNanley, Elizabeth M Cooper, Allison W McIntyre, Frank Witter, Z Leah Harris, Kimberly O O'Brien. Maternal Vitamin D and Calcium Interact to Impact Fetal Skeletal Growth In-Utero in Pregnant Adolescents.

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ABSTRACT

Background: Maternal calcium (Ca) intake and vitamin D status may impact fetal bone development.

Objective: This study was designed to examine relationships between maternal Ca intake, 25(OH)D status and fetal bone growth across pregnancy.

Design: This was a prospective longitudinal design. Maternal 25(OH)D, PTH, and $1,25(OH)_2D$ were determined at mid-gestation (~26 weeks) and at delivery in 171 adolescents (≤ 18y). Dietary recalls and fetal sonograms were performed up to three times across gestation; fetal femur and humerus Z-scores were generated.

Results: Fetal femur and humerus Z-scores and neonatal birth length were significantly greater ($p < 0.03$) in adolescents consuming ≥1050 mg vs. those consuming <1050 mg Ca/day. Maternal 25(OH)D > 20 ng/mL was significantly positively associated with fetal femur and humerus Z-scores ($p < 0.01$). When controlling for maternal smoking, height, race, weight gain, and gestational age, these relationships remained significant. Interactions between Ca intake and 25(OH)D were evident. Calcium intake was only associated with fetal femur Z-scores and birth length when maternal 25(OH)D was ≤ 20 ng/mL ($p < 0.05$). Similarly, maternal 25(OH)D was only associated with fetal femur and humerus Z-scores when maternal Ca intake was < 1050 mg/day ($p < 0.03$).

Conclusions: Optimal Ca intake and adequate maternal vitamin D status are both needed to optimize fetal bone growth. Interactions between these nutrients were evident when either Ca or vitamin D status was limited. Optimizing maternal Ca intake and/or vitamin D status during pregnancy status may have a positive impact on fetal skeletal development in pregnant adolescents.

INTRODUCTION

The prevalence of vitamin D deficiency (25(OH)D ≤ 10 mg/mL) in pregnant women residing at northern latitudes has been reported to range between 21 to 50% (1-3); an even larger number of women are vitamin D insufficient (25(OH)D ≤ 20 ng/mL) (3). This high prevalence may be of concern given the number of reports linking insufficient vitamin D status during pregnancy with detrimental effects on fetal growth and development, and subsequent risk of chronic diseases (4). Both vitamin D and Ca are needed to fully mineralize the developing skeleton. Thus low Ca intake, especially when combined with insufficient vitamin D status, may limit fetal bone growth and mineralization (5).

To date, maternal 25(OH)D status during pregnancy has been linked to infant birth weight (6) and adverse neonatal bone outcomes, including impaired fetal femoral development (7), reduced bone mineral content (BMC), and reduced neonatal bone density at birth (8;9). The bone alterations observed *in-utero* may impact subsequent pediatric bone mineralization as children whose mothers had low 25(OH)D status during pregnancy have reduced BMC at 9 y of age (10).

Like maternal vitamin D status, maternal Ca intake impacts fetal skeletal development. Supplemental Ca provided to women with low Ca intake (< 600 mg/day) resulted in increased bone density and total body BMC in the neonate (11;12). In pregnant adolescents, maternal dairy intake has been positively associated with increased fetal femur length (13) and milk intake has been associated with infant total body Ca (14). In adults, maternal milk and vitamin D intake have been found to be significantly associated with infant birth weight (15). Additionally, maternal milk intake during pregnancy has been linked to subsequent spinal bone mineral density (BMD) of offspring at 16 y of age (16). In food-based Ca supplementation studies (i.e.

dairy products), it is difficult to distinguish whether it is the Ca and/or vitamin D or other components of dairy products that are impacting neonatal bone outcomes. Similarly, many studies fail to control for possible interactions between maternal Ca intake and vitamin D status when investigating the impact of maternal status of these nutrients on neonatal outcomes. These interactions are biologically plausible given that the active form of vitamin D, $1,25(OH)_2D$, is generated from 25(OH)D and this hormone stimulates active intestinal Ca absorption particularly when Ca intakes are low. At present, few studies addressing the effects of maternal Ca intake and vitamin D status on fetal development have also measured circulating $1,25(OH)_2D$. This is of concern as there is a growing awareness that local paracrine/autocrine effects of $1,25(OH)_2D$ are influenced by circulating 25(OH)D (17).

Pregnancy during adolescence may result in a competition for nutrients between the mother and her developing fetus, placing both at risk for adverse outcomes (18). To address possible associations and interactions between maternal Ca intake and 25(OH)D status with fetal bone growth, we followed a large cohort of pregnant adolescents across gestation obtaining measures of fetal skeletal development *in-utero* in relation to maternal Ca intake, calcitropic hormones, and 25(OH)D status.

MATERIALS AND METHODS

Participants and Study Procedures

A cohort of 171 pregnant adolescents (≤ 18 y) was recruited to participate in a prospective longitudinal study designed to characterize changes in maternal bone quality and fetal bone growth across gestation. Thirteen participants were recruited from the Maternity Center East Clinic in Baltimore, MD, and followed until delivery at Johns Hopkins Hospital (Baltimore, MD). The remainder of the adolescents (n = 158) were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY. Pregnant adolescents were eligible to participate if they were between 12 and 30 weeks gestation at entry into the study, were otherwise healthy, and were carrying a single fetus. Exclusion criteria included known medical complications including: HIV infection, diabetes, diagnosed eating disorders, or malabsorption diseases. Informed written consent was obtained from all participants, and study procedures were approved by the Institutional Review Boards of the University of Rochester, Cornell University, and Johns Hopkins University. Maternal pre-pregnancy weight and smoking history were self-reported. Smoking status was self-reported as never, previously, or currently smoking and information on the number of cigarettes smoked per day was obtained in those that were currently smoking. Maternal ethnicity (Hispanic or Non-Hispanic) and race (African-American, Caucasian, or Other) were self reported. Neonatal race was classified as African American if the mother reported both herself and the father as African American; as Caucasian if the mother reported both herself and the father as Caucasian; or biracial if maternal and paternal race differed. Infant ethnicity was similarly classified as either: Hispanic, Non-Hispanic, or multi-ethnic. Data on markers of bone turnover and OPG concentrations (Appendix 11) and maternal calcitropic hormone concentrations (Chapter 2) in this cohort have been previously published.

Each adolescent attended up to three study visits across pregnancy, roughly timed to coincide with early, mid, and late gestation. At each visit maternal anthropometric measures were recorded and a 24-hour dietary recall was administered by study personnel using food models to help estimate portion sizes. All 24-hour recalls were analyzed by a registered dietitian using the Nutrition Data System for Research (NDSR: University of Minnesota, Minneapolis, MN, versions 2006, 2008, and 2009) at the University of Rochester CTRC. Tertiles of dietary Ca intake were defined based on observed Ca intakes.

Up to three times across pregnancy, both standard fetal biometry measures (femur length, biparietal diameter, abdominal circumference and head circumference) and humerus length were recorded by certified sonographers. Fetal humerus length Z-scores were calculated from published curves generated by Chitty et al. in adult women (19). Fetal femur length Z-scores were generated from equations previously published by our laboratory, derived from a large cohort (n = 929) of African-American pregnant adolescents in Baltimore, MD, USA (13). The curves generated for fetal femur growth in these pregnant adolescents are comparable to similar curve fits generated from data obtained in adult women (13;19). At birth, infant weight, length, and head circumference were recorded by clinical staff, according to standard procedures.

Biochemical analyses

Maternal blood (10 mL) was obtained at mid-gestation (~26 weeks) and again at delivery. All blood samples were allowed to clot at room temperature, before serum was separated by centrifugation. An aliquot of serum was immediately sent to Quest laboratories for assessment of 25(OH)D using the Diasorin RIA (Diasorin Inc, Stillwater, MN). This laboratory participates in the vitamin D External Quality Assessment Scheme (DEQAS) as a means of quality assurance. Serum collected for other biochemical assessment was stored at -80^0C until analysis.

Season of each blood collection was classified as winter (November-February), spring (March-April), summer (May-August), or autumn (September-October) using seasonal classifications for the northeast United States (20). Vitamin D insufficiency was defined as 25(OH)D ≤ 20 ng/mL in accordance with recent 2010 IOM guidelines (21). After a high prevalence of vitamin D insufficiency was observed in the first 45 study participants, all subsequent participants found to be vitamin D insufficient at mid-gestation were provided with an additional 400 IU of vitamin D_3 at their next prenatal visit, and were instructed to take one pill daily over the remainder of gestation. Compliance with all prenatal supplements provided was queried by self-report at each study visit. Calcitriol ($1,25(OH)_2D$) was analyzed at Boston University in the laboratory of Dr. Michael Holick (Boston, MA) using an in-house thymus receptor binding assay as previously described (22). Due to the lack of standardized reference ranges for calcitriol concentrations across pregnancy, adolescents were classified as exhibiting $1,25(OH)_2D$ concentrations above or below the mean $1,25(OH)_2D$ value both at mid-gestation and at delivery. Intact parathyroid hormone (PTH) was analyzed using a commercially available ELISA (DSL Laboratories, Webster, TX). Elevated PTH was defined if concentrations were ≥ 46 pg/mL (23).

Statistical Analyses

Analyses were performed using SAS 9.2 and JMP 8.0 (SAS Institute Inc, Cary, NC). Results are reported as the mean ± standard deviation (SD) unless otherwise stated. Paired t-tests or non-parametric tests were used to assess changes in hormones across gestation within subjects. Independent t-tests or ANOVA were used to determine if normally distributed variables differed by race, season, or categories of vitamin D status and Ca intake; the Wilcoxon Rank sum test was utilized for nonparametric data.

Simple linear regression was used to explore relationships among Ca intake, 25(OH)D, PTH, and 1,25$(OH)_2$D and fetal skeletal growth. Multiple linear regression was used to control for covariates and interactions between variables and to model statistical predictors of measures of fetal skeletal growth. Previous research had identified maternal height, pre-pregnancy BMI, and dairy intake as predictors of fetal femur length in a group of 350 African American adolescents (13). As such, we initially considered maternal height and pre-pregnancy BMI, as well as infant sex, season of delivery, maternal race, weight gain, smoking status, chronological age, and gynecological age as potential predictors of fetal femur and humerus Z-scores and neonatal birth length. Using simple linear regression, maternal smoking status, race, height, weight gain, and gestational age at delivery (for birth length) were identified as potential statistical predictors ($p < 0.20$) of fetal femur and humerus Z-score and/or neonatal birth length. These variables were controlled for as covariates in the generated models of fetal bone Z-scores and birth length.

Interactions between maternal Ca intake and vitamin D status on fetal and neonatal bone outcomes were assessed statistically. Relationships between maternal Ca intake and bone outcomes were also analyzed stratified by categories of vitamin D status in order to describe the nature of interactions between Ca and vitamin D status (≤ 20 or > 20 ng/mL) on fetal and neonatal bone outcomes. Similarly, relationships between maternal vitamin D status and fetal bone outcomes were analyzed when stratified by categories of Ca intake (tertiles or < 1050 vs. ≥ 1050), in order to assess potential interactions between the two nutrients.

Using published means and standard deviations for fetal femur length reported in pregnant adolescents (13), we determined that a sample size of 146 participants would provide us with sufficient power (0.85) to characterize the mean fetal femur lengths in this population and to

obtain a minimal detectable difference of 0.25 Z-scores from expected fetal bone measures, with an alpha level of 0.05. We over-recruited by 14% to allow for missing data and possible subject loss to follow-up due to the known increased risk of fetal death *in-utero* and preterm birth in this age group. Variables were tested for normality using the Shapiro Wilks test. Non-normally distributed variables were log transformed as necessary to ensure normality of the residuals. P values < 0.05 were considered significant, and P values between 0.05 and 0.10 were considered trends of interest.

RESULTS

Subject Characteristics

Characteristics of the adolescents are presented in **Table 3.1**.

Table 3.1. Characteristics of Pregnant Adolescents Enrolled

Maternal Characteristics	Mean ± SD (n)
Total Recruitment	171
Age at Enrollment (years)	17.1 ± 1.1 (170)
Racial Group: African American	66.7% (114)
Caucasians	33.3% (57)
Ethnicity: Hispanic	23.7% (39)
Non-Hispanic	76.2% (125)
Parity ≥ 1	8.2% (14/170)
Smoking at Entry into study	10.0% (17/170)
Pre-Pregnancy BMI (kg/m^2)	24.7 ± 5.5 (165)
Weight Gain (lbs)	37.1 ± 1.5 (150)
Dietary Ca Intake (mg/day)[1]	917 ± 416 (163)
Dietary Vitamin D intake (IU/day)[1]	216 ± 135 (163)
Gestational Age at Delivery (weeks)	39.2 ± 2.9 (165)
Maternal 25(OH)D at Delivery (ng/mL)	21.9 ± 11.0 (168)
Maternal $1,25(OH)_2D$ at Delivery (pg/mL)	106.2 ± 30.6 (96)
Maternal PTH at Delivery (pg/mL)	45.1 ± 29.4 (80)

[1] Intakes presented are the mean of all 24-hour recalls administered over pregnancy (up to three for each participant)

Birth data was missing or unavailable in nine adolescents: of these three (1.8%) suffered a fetal death *in-utero*, while six adolescents either dropped out of the study prior to delivery or delivered at another hospital, so birth data was not obtained. Among neonates assessed at delivery, birth length was recorded in the medial chart of 153 (94.4%) infants. In 163 adolescents (95.3%), at least one dietary assessment was obtained during pregnancy, and 25(OH)D was assessed at least once across gestation in 168 (98.2%) study participants. Gestational age at delivery ranged from 20.7 to 43.0 weeks; 8.5% of births were premature (< 37 weeks gestation). Two of the 171 adolescents self-identified their race as American Indian. None of the study results differed if these two adolescents were excluded from analyses or combined with either the African American or Caucasian cohort. To avoid eliminating data from these two adolescents, they were grouped with the African American cohort in order to collapse maternal race into a bivariate variable.

In adolescents with multiple 24-hour recall data, reported Ca, vitamin D, and caloric intake did not significantly differ between study visits, so the mean of all visits for each participant was used for all subsequent analyses. Dietary Ca intake ranged from 257 to 3220 mg (**Table 3.1**), with only 29.4% of adolescents meeting the EAR (1100 mg/day) (21), and 15.3% of adolescents meeting the RDA (1300 mg/day). Maternal vitamin D intake was highly correlated with Ca intake ($p < 0.0001$, $R^2 = 0.40$). Because maternal 25(OH)D concentrations at mid-gestation did not significantly differ from concentrations at delivery ($p = 0.64$), when 25(OH)D was not assessed at delivery ($n = 31$ out of 168), the 25(OH)D concentration obtained at mid-gestation was substituted for this measure in order to have a measure of vitamin D status from each participant (**Table 3.1**). Maternal vitamin D and Ca intake were both positively correlated with maternal 25(OH)D ($p = 0.002$, $R^2 = 0.06$ and $p = 0.004$, $R^2 = 0.05$, respectively, $n = 162$).

Vitamin D insufficiency (25(OH)D ≤ 20 ng/mL), was present in 47.6% of adolescents and 52.8% of neonates at birth. Supplemental vitamin D_3 (400 IU/day) was provided to 56% of teens whose mid-gestation 25(OH)D concentration was ≤ 20 ng/mL. Those who were vitamin D insufficient and did not receive supplements either joined the study before the supplements were provided (n = 19), failed to return to the clinic to receive the supplements (n = 15), or refused to accept them (n = 4). Vitamin D insufficient adolescents who received supplements began supplementation on average at 30.3 ± 3.5 weeks gestation. Self-reported data indicated that 35.8% of these adolescents consumed the vitamin D supplement at least two times per week, and only 26.4% of adolescents self-reported that they consumed these supplements daily. The change in 25(OH)D concentrations from mid-gestation to delivery observed in those who received supplements (3.2 ng/mL, n = 45) was significantly different than the change observed in those who were not supplemented (-2.3 ng/mL, n = 90, p = 0.0002). Despite this increase in 25(OH)D, teens who received vitamin D supplements still had significantly lower 25(OH)D concentrations (17.4 ng/mL vs. 23.6 ng/mL, p = 0.001) and a higher prevalence of vitamin D insufficiency (63.3% vs. 44.4%, p = 0.03) at delivery compared with teens who did not receive supplements. When considered as a potential covariate, maternal receipt of vitamin D supplements was not significant in any models of fetal and neonatal skeletal outcomes.

Birth weight and length did not differ by maternal or infant race, infant sex, season of delivery, or receipt of vitamin D supplements. Data from at least one sonogram was available in all but two adolescents. The femur and humerus lengths from the last sonogram measure obtained (at 33.8 ± 4.0 weeks gestation) were used to generate gestational age-specific fetal bone Z-scores. Humerus measures were not obtained from two adolescents due to position of the fetus at the time of measurement. Fetal femur and humerus length Z-scores were both significantly

below zero ($p < 0.0001$, $n = 169$ and $p = 0.009$, $n = 167$) and did not differ by maternal race (**Table 3.2**), infant race, infant sex, season of delivery, or receipt of vitamin D supplements, although there was a trend for higher fetal femur Z-scores in African American verses Caucasian adolescents ($p = 0.054$). Fetal femur length Z-score in non-smoking adolescents was significantly more positive, by 0.424 SD, than in adolescents who were smoking ($p = 0.030$).

Table 3.2. Fetal and Neonatal Outcomes as a Function of Maternal Race[1]

Neonatal Characteristics	**All Adolescents**	**African American**	**Caucasian**	**p**
Birth Weight (g)	3238 ± 586 (162)	3210 ± 599 (108)	3293 ± 561 (54)	0.300
Birth Length (cm)	51.0 ± 2.6 (153)	51.0 ± 2.6 (102)	51.2 ± 2.2 (51)	0.932
Fetal Femur Length Z-Score	-0.540 ± 0.918 (169)	-0.446 ± 0.917 (114)	-0.734 ± 0.897 (55)	0.054
Fetal Humerus Length Z-Score	-0.201 ± 0.971 (167)	-0.197 ± 1.024 (112)	-0.207 ± 0.865 (55)	0.950

[1] Data presented as mean ± SD with sample size in parentheses
p –values are for comparison between neonates born to African American vs. Caucasian adolescents.

Maternal $1,25(OH)_2D$ and PTH concentrations at mid-gestation and delivery were not significantly related to fetal femur, or humerus length Z-scores, or neonatal birth length. Furthermore, fetal femur and humerus Z-scores and neonatal birth length did not differ between

adolescents with elevated PTH vs. those with PTH < 46 pg/mL at either time point, or between adolescents with 1,25$(OH)_2$D concentrations above vs. below the mean concentration observed at mid-gestation (116.9 pg/mL) or delivery (106.2 pg/mL).

Maternal Ca Intake and Fetal Bone Outcomes

Maternal Ca intake, as a linear variable, was not associated with fetal femur or humerus Z-score. When the cohort was divided into tertiles of Ca intake (< 653; < 1066; ≥ 1066 mg/day), those in the highest tertile had a significantly more positive fetal femur length Z-score ($p < 0.040$), when compared to those in the lower two tertiles (**Figure 3.1A**). The same trend was present for fetal humerus length Z-score but this difference only approached significance ($p < 0.070$). A marked difference in fetal bone Z-scores was evident between the second and third tertile of Ca intake (653 to 1066 mg/day). To further define the Ca intake at which significant increases in fetal bone Z-scores became evident, possible differences in fetal bone Z-scores were evaluated at 50 mg intervals across the Ca intake range of 653-1066 mg/day. Using this approach, a Ca intake of ≥ 1050 mg/day was identified as the intake at which differences in both fetal femur length Z-score (-0.319 vs. -0.680, $p = 0.020$, $n = 161$) and fetal humerus length Z-score (0.003 vs. -0.337; $p = 0.024$, $n = 159$) became evident (**Figure 3.1B**). The raw difference in fetal femur Z-score observed in those consuming < 1050 mg/day was equivalent to a 0.98 mm shorter femur at 33.8 weeks, which represents 1.5% of the mean predicted femur length at that week gestation. The raw difference in fetal humerus Z-score observed in those consuming < 1050 mg/d was equivalent to a 0.92 mm shorter humerus at 33.8 weeks, which represents 1.6% of predicted humerus length at that week gestation.

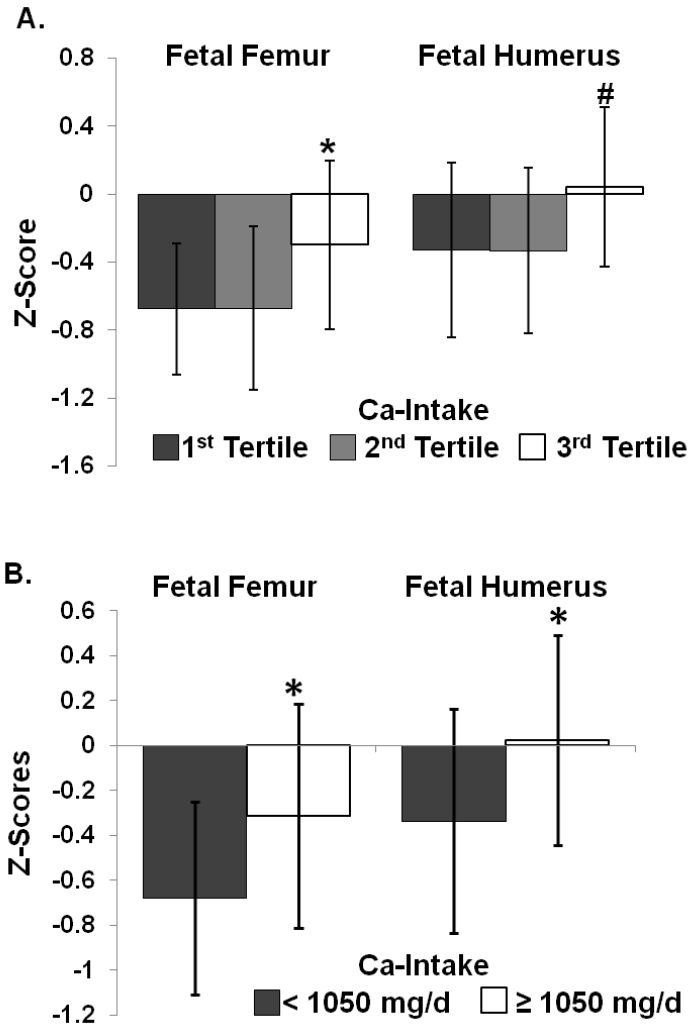

Figure 3.1 Fetal Femur and Humerus Z-scores Differ by Maternal Calcium Intake
Fetal femur and humerus length were evaluated by sonogram at 33.8 ± 4.0 weeks gestation in 169 pregnant adolescents and Z-scores were calculated for each measure. Ca intake was categorized into tertiles: 1st < 653 mg/day; 2nd = 653 - 1066 mg/day; 3rd > 1066 mg/day. A: Adolescents consuming within the third tertile of Ca intake had better fetal femur length Z-scores ($p < 0.039$) and tended to have better fetal humerus length Z-scores (# $p < 0.070$) compared to those in the first and second Ca tertile. B: Between the second and third tertile of Ca intakes, the Ca intake of ≥ 1050 mg/day was identified as the intake at which significant differences in fetal femur ($p = 0.020$) and humerus ($p = 0.024$) Z-scores became evident.

Multiple regression models of fetal femur and humerus Z-scores were generated to test the main effect of maternal Ca intake after controlling for the identified covariates: maternal

smoking status, race, height, and weight gain. After controlling for these maternal characteristics, maternal Ca intake ≥ 1050 mg/day remained a significant predictor of fetal femur Z-score (parameter estimate = 0.009) and was associated with a 0.196 increase in fetal femur Z-score. Maternal Ca intake ≥ 1050 mg/day explained an additional 4% of the variation in femur Z-score (i.e, increased the R^2 of the model from 0.031 to 0.068). Similarly, when controlling for the identified covariates, maternal Ca intake ≥ 1050 mg/day also remained a significant predictor of fetal humerus length Z-scores (parameter estimate = 0.026). In step-wise regression, all other covariates dropped out of the model of fetal humerus Z-score, and maternal Ca intake ≥ 1050 mg/day was associated with a 0.180 increase in fetal humerus Z-score and predicted 3.2% of the variation in this measure.

Maternal 25(OH)D status and Fetal Bone Outcomes

When analyzed as a continuous variable, maternal dietary vitamin D intake was significantly positively associated with fetal humerus Z-score ($p = 0.015$, $R^2 = 0.037$, $n = 158$), but not with any other fetal/neonatal outcomes. Maternal 25(OH)D is a biomarker for vitamin D status, and when analyzed as a categorical variable, pregnant adolescents with sufficient 25(OH)D status (> 20 ng/mL) had significantly higher fetal femur and fetal humerus length Z-scores compared to measures obtained in vitamin D insufficient adolescents (-0.321 vs. -0.746, $p = 0.003$, $n = 166$ and 0.012 vs. -0.403, $p = 0.006$, $n = 164$). After controlling for maternal smoking status, race, height, and weight gain, maternal 25(OH)D sufficiency remained a significant predictor of fetal femur length Z-score (parameter estimate = 0.001) and was associated with a 0.225 increase in fetal femur Z-score. Maternal 25(OH)D > 20 ng/mL explained an additional 6% of the variation in femur Z-score (i.e. increased the R^2 of the model from 0.031 to 0.089). Maternal 25(OH)D sufficiency also remained a significant predictor of

fetal humerus length Z-scores when controlling for identified covariates (parameter estimate = 0.006). In step-wise regression, all other covariates dropped out of the model of fetal humerus Z-score, and maternal 25(OH)D sufficiency was associated with a 0.208 increase in fetal humerus Z-score and predicted 4.5% of the variation in this measure.

Interaction between Maternal Ca intake and 25(OH)D status on Fetal Bone Outcomes

Because both maternal Ca intake ≥ 1050 mg/day and maternal 25(OH)D > 20 ng/mL were individually associated with fetal femur and humerus Z-scores, both of these variables, along with an interaction term were entered into a model to test for possible interactions between the effects of maternal 25(OH)D > 20 ng/mL and maternal Ca intake ≥ 1050 mg/day on fetal bone length. In this combined model of fetal femur length Z-score, the main effect of Ca ≥ 1050 mg/day, the main effect of 25(OH)D sufficiency, and the interaction term between Ca intake and 25(OH)D all remained significant ($p < 0.05$) (**Table 3.3**). In the best model identified for humerus length Z-score, the main effect of maternal Ca intake ≥ 1050 was borderline significant ($p < 0.10$), the main effect of 25(OH)D sufficiency remained significant, and the interaction term was not significant. These models are summarized in **Table 3.3**.

Table 3.3. Maternal Ca Intake and Vitamin D Sufficiency are Determinants of Fetal Femur and Humerus Z-scores in Pregnant Adolescents

Covariate[1]	**Fetal Femur Z-Score**		**Fetal Humerus Z-score**	
	β ± SE[2]	p	β ± SE[2]	p
25(OH)D < 20 ng/mL	-0.1462 ± 0.0738	0.049	-0.1753 ± 0.0784	0.027
Ca intake < 1050 mg/day	- 0.1655 ± 0.0738	0.026	-0.1341 ± 0.0808	0.099
25(OH)D X Ca intake[3]	-0.1628 ± 0.0739	0.029	-	-
Maternal Race (AA)	0.1744 ± 0.0741	0.020	-	-
Maternal Height (m)	0.0203 ± 0.0100	0.042	0.0166 ± 0.0109	0.130
p	< 0.0001		0.007	
R^2	0.125		0.057	
n	160		158	

AA = African American

[1] In step-wise regression, maternal weight gain and smoking status were not significant in models of either bone Z-score. In step-wise selection, the interaction term and race were dropped from the model of fetal humerus Z-score.

[2] β ± Standard Error

[3] Interaction term between maternal Ca intake and 25(OH)D status

To further investigate the nature of the interaction between maternal Ca intake ≥ 1050 mg/day and 25(OH)D sufficiency, the initial models of fetal femur and humerus length Z-scores that included Ca intake as a bivariate variable ≥ 1050 mg/day were run separately in adolescents with maternal 25(OH)D ≤ 20 ng/mL, and in adolescents with 25(OH)D > 20 ng/mL. In both the models of fetal femur and humerus Z-score, Ca intake remained a significant covariate only in adolescents with 25(OH)D ≤ 20 ng/mL. **Figure 3.2** presents the effect of maternal Ca intake ≥

1050 mg/day on fetal femur and humerus Z-scores separately in adolescents who were 25(OH)D sufficient and 25(OH)D insufficient.

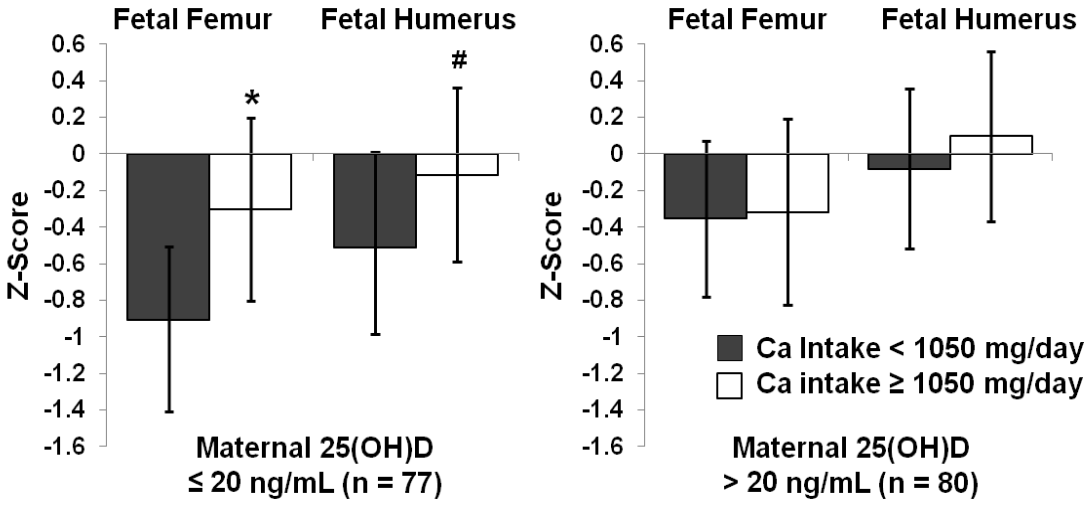

Figure 3.2 Fetal Femur and Humerus Z-scores are Higher in Adolescents with Ca Intakes ≥ 1050 mg/day when Maternal 25(OH)D is Insufficient
Third trimester (33.8 ± 4.0 weeks gestation) fetal femur and humerus lengths were measured and converted into Z-scores. Maternal dietary Ca intake and 25(OH)D status interacted to impact bone length outcomes. In adolescents with 25(OH)D ≤ 20 ng/mL, those who consumed ≥ 1050 mg Ca/day had significantly higher fetal femur Z-Score (* $p = 0.019$) and higher fetal humerus Z-score (# $p = 0.116$). Among adolescents who were 25(OH)D sufficient, there was no significant difference in fetal Z-scores between adolescents who consumed above or below 1050 mg Ca/day.

Similarly, when the initial models of fetal femur and humerus length Z-scores that included 25(OH)D sufficiency were run separately in adolescents consuming above and below 1050 mg Ca/day, 25(OH)D sufficiency remained a significant predictor only in adolescents consuming < 1050 mg Ca/day. **Figure 3.3** presents the effect of maternal 25(OH)D sufficiency on fetal bone Z-scores separately in adolescents consuming Ca intakes above and below 1050 mg/day.

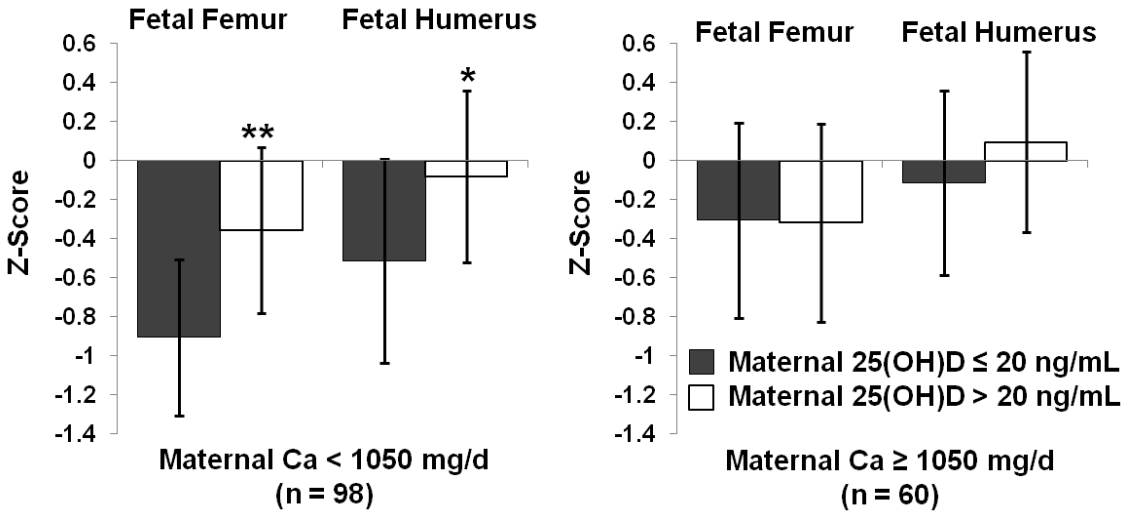

Figure 3.3 Fetal Femur and Humerus Z-scores are Higher in Adolescents with Sufficient 25(OH)D when Maternal Ca Intakes < 1050 mg/day
An interaction between maternal 25(OH)D and maternal Ca intake was observed such that, in adolescents consuming < 1050 mg/day Ca, those with 25(OH)D > 20 ng/mL had significantly higher fetal femur (** $p = 0.001$) and humerus Z-scores (* $p = 0.030$). Among adolescents who consumed ≥ 1050 mg Ca/day, there were no significant differences in fetal long bone Z-scores between adolescents who were 25(OH)D sufficient vs. insufficient.

The combined effect of 25(OH)D status and Ca intake on Fetal Bone Outcomes

The above interactions suggested that both adequate maternal Ca intake (≥ 1050 mg/day) or sufficient 25(OH)D status were capable of compensating when intake or status of the other nutrient was limited. To further explore this possibility, we classified teens into two groupings according to Ca intake and vitamin D status as "Ca/D insufficient" if both maternal 25(OH)D ≤ 20 ng/mL and Ca intake < 1050 mg/day, and all others were classified as "Ca/D sufficient". Adolescents classified as Ca/D sufficient had significantly more positive fetal femur ($p < 0.001$, $n = 160$) and humerus ($p = 0.002$, $n = 158$) Z-scores in comparison to measures obtained in the Ca/D insufficient group (**Figure 3.4**). When controlling for maternal smoking, height, race, and weight gain, Ca/D sufficiency remained a significant determinant of both fetal femur (parameter

estimate $p < 0.0001$) and humerus (parameter estimate $p = 0.002$) Z-scores explained an additional 10.5% of the variation in fetal femur Z-score and an additional 5.3% of the variation in fetal humerus Z-score. The improvement associated with moving from the Ca/D insufficient to Ca/D sufficient group, by improving either Ca intake or 25(OH)D status, was 0.314 SD in fetal femur Z-score and 0.249 SD in fetal humerus Z-score.

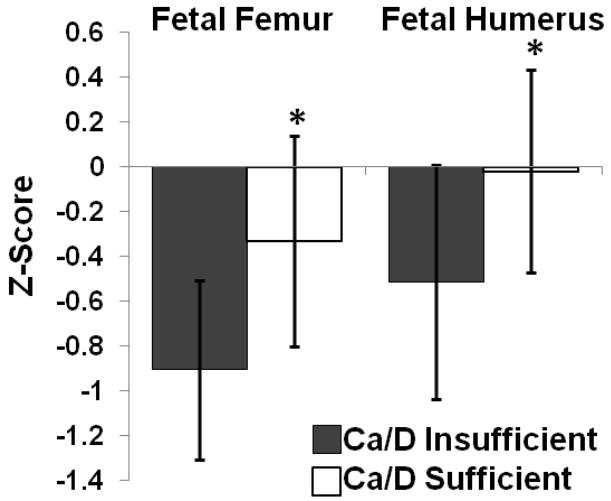

Figure 3.4 Fetal Femur and Humerus Z-scores are Higher in Adolescents with 25(OH)D > 20 ng/mL and/or Ca Intakes ≥ 1050 mg/day Compared to those with Insufficient Ca Intakes and Insufficient 25(OH)D
Adolescents consuming Ca intakes < 1050 mg/day who also had 25(OH)D ≤ 20 ng/mL (Ca/D insufficient) were compared to adolescents with Ca intake ≥ 1050 mg/day and/or 25(OH)D > 20 ng/mL (Ca/D sufficient). Adolescents within the "Ca/D sufficient" category (n = 102) had higher fetal femur and humerus Z-scores (* $p < 0.0001$ and $p = 0.003$, respectively) when compared to those in the "Ca/D insufficient" category (n = 60).

There were no differences in either bone Z-score value between adolescents with either an adequate maternal Ca intake (≥ 1050 mg/day) or a sufficient 25(OH)D value vs. adolescents who were adequate/sufficient in both Ca and 25(OH)D.

The combined effect of 25(OH)D status and Ca intake on Neonatal Birth Length

The observed effects of maternal Ca intake on fetal bone length *in-utero* were also evident at birth using recorded measures of neonatal birth length. Adolescents in the highest tertile of Ca intake delivered neonates with a significantly longer mean birth length ($p < 0.020$) in comparison to the mean observed in adolescents consuming in the lower two tertiles (**Figure 3.5A**). Similarly, adolescents consuming ≥ 1050 mg Ca/day delivered neonates that were 1.03 cm longer at birth than infants delivered to adolescents consuming < 1050 mg Ca/day ($p = 0.028$, $n = 147$; **Figure 3.5B**). In a multiple regression model of neonatal birth length (that controlled for gestational age at delivery, maternal smoking status, race, height and weight gain), maternal Ca intake (as a linear variable) remained a significant predictor of neonatal birth length (parameter estimate $p = 0.041$), and explained an additional 3% of the variation in birth length. In this model, every 100 mg increase in Ca intake was associated with a 0.12 cm (95% CI: 0.02 – 0.21 cm) increase in birth length.

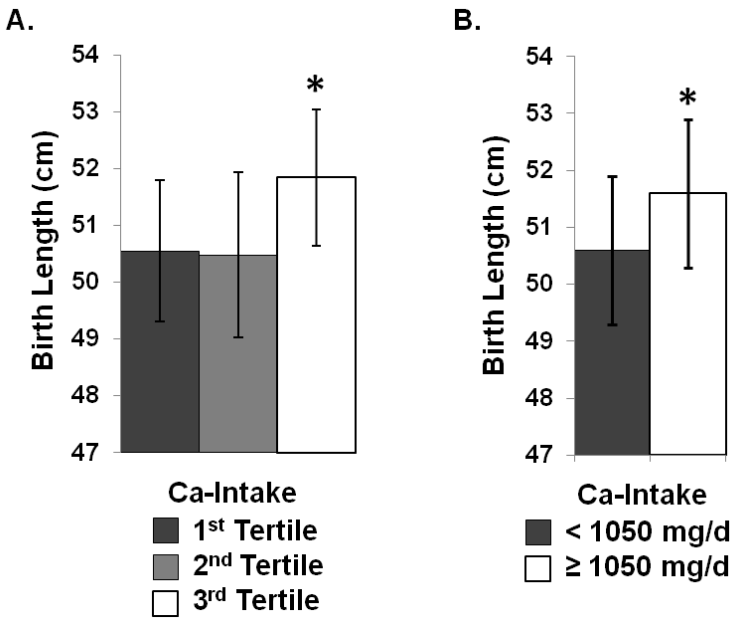

Figure 3.5 Neonatal Birth Length Differs by Maternal Ca Intake
Birth length was measured in 153 neonates born to adolescents (≤ 18 y of age). Maternal Ca intake was categorized into tertiles: 1st < 653 mg/day; 2nd = 653 - 1066 mg/day; 3rd > 1066 mg/day, and also categorized as above or below the identified Ca intake at which differences in fetal long bone Z-scores were evident (1050 mg/day). A: Adolescents consuming within the third tertile of Ca intake delivered infants that were significantly longer at birth (* $p < 0.015$). B: Adolescents consuming ≥ 1050 mg of Ca/day delivered infants that were significantly longer at birth (* $p = 0.035$).

In contrast to the significant associations found between neonatal birth length and maternal Ca intake, maternal 25(OH)D status was not significantly correlated with birth length. While a main effect of maternal 25(OH)D status was not evident, an interaction between maternal Ca intake and maternal 25(OH)D sufficiency was observed. When the initial model of birth length that included Ca intake as a linear variable was run separately in adolescents with maternal 25(OH)D ≤ 20 ng/mL, and in adolescents with 25(OH)D > 20 ng/mL, Ca intake remained a significant covariate only in adolescents with 25(OH)D ≤ 20 ng/mL. Furthermore,

when maternal Ca intake and 25(OH)D status were combined and grouped as Ca/D insufficient or Ca/D sufficient (as described above), this Ca/D sufficiency variable remained borderline significant (parameter estimate $p = 0.074$) in the model of birth length, increased the R^2 of the model from 0.035 to 0.038, and was associated with a 0.34 cm increase in birth length.

DISCUSSION

In these pregnant adolescents, both maternal 25(OH)D status and dietary Ca intake influenced fetal bone growth. A significant interaction was evident between these two nutrients with higher Ca intakes compensating for suboptimal vitamin D status and vice versa. These interactions are biologically feasible given the known role of vitamin D on active Ca absorption. The relationships and interactions detected *in-utero* remained evident at delivery, as evidenced by significant differences in neonatal birth length.

Dietary Ca intake had a significant effect on fetal bone. Significantly longer fetal femur and humerus lengths were evident in adolescents consuming Ca intakes ≥ 1050 mg/day. The 1050 mg/d Ca intake at which these significant differences in bone outcomes became apparent is similar to the 2010 EAR (1100 mg/day) set for this age group by the IOM (21). The DRI recommendations for Ca intake for pregnant adolescents do not differ from recommendations set for non-pregnant adolescent females. The recommended intakes are based on achieving maximum Ca retention during adolescent years (21). When undertaking analyses stratified by vitamin D status, we chose to use the concentration identified by the IOM that corresponds with the RDA for vitamin D sufficiency (25(OH)D ≤ 20 ng/mL). For Ca intake, we chose to use the Ca intake cut-point identified in our data (1050 mg/day) for stratified analyses, as opposed to the RDA of 1300 mg for two reasons. Firstly, this intake was identified based on its association with functional fetal and neonatal skeletal outcomes (fetal femur and humerus Z-scores and birth length), which the DRI does not assess. Secondly, use of the RDA for Ca intake (1300 mg/day) was impractical for making statistically sound comparisons between groups as only 15.3% of adolescents in this study actually consumed ≥ 1300 mg Ca/day.

The raw difference in fetal femur Z-score observed in those consuming < 1050 mg/d was 0.36 Z-scores. Other in-vivo studies have found that maternal dairy intake is significantly related to fetal femur length in pregnant adolescents (13). Similar nutrient driven gains in fetal femur length (0.24 Z-scores at 38 weeks) have also been documented in disadvantaged Peruvian women provided daily 25 mg zinc supplements (24). Our observed deficit in fetal humerus (0.34 Z-scores) was similar to that observed for the femur. Biological plausibility for a role of Ca in long bone elongation is supported by several in-vitro studies in animal cells that have shown that extracellular Ca^{2+} concentrations and Ca^{2+}-sensing receptor activation in developing fetal bone induces type X collagen synthesis, increases rates of development and differentiation of growth plate chondrocytes, and increases the height of the growth plate hypertrophic zone (25-27).

Similar to the findings evident for Ca intake, pregnant adolescents with sufficient 25(OH)D exhibited more positive fetal femur and humerus Z-scores (by 0.42 SD). These results substantiate findings in adult pregnant women that have also found low maternal 25(OH)D to be associated with adverse fetal and neonatal bone outcomes (7;9;28). While many mechanistic studies have focused on the role of $1,25(OH)_2D$ on regulation of endochondral bone development (29), it is interesting to note that we found no significant relationships between $1,25(OH)_2D$ and fetal bone Z-scores. Increasing attention has been paid to the importance of local production and action of $1,25(OH)_2D$ on bone growth (30), where availability of the 25(OH)D substrate may impact rates of $1,25(OH)_2D$ synthesis. Thus, if systemic circulating $1,25(OH)_2D$ concentrations are not reflective of local production, we may not expect to see on overall association between circulating $1,25(OH)_2D$ and fetal bone. The high prevalence of vitamin D insufficiency observed in these adolescents (48%) is similar to that reported among pregnant adult women in the United States (31).

A significant interaction between dietary Ca intake and 25(OH)D status was evident, such that maternal Ca intake ≥ 1050 mg/day and maternal 25(OH)D ≤ 20 ng/mL were only significantly associated with fetal femur and humerus Z-scores when the other nutrient was inadequate. This suggests that increases in dietary Ca intake and/or improvements in maternal vitamin D status can offset deficits when the other nutrient is limited. Similar interactions have been described in non-pregnant adolescent females (age 11-16 years, n = 211) in whom reduced areal bone mineral density Z-scores were observed in girls that had both low dietary Ca intake (< 600 mg/day) and low 25(OH)D status (≤ 16 ng/mL) when compared to girls who exhibited either higher dietary Ca intakes and/or vitamin D status (32). **Figure 3.6** below represents a conceptual diagram of the interaction between maternal Ca intake and 25(OH)D status on fetal bone development (adapted from (33)). Ideally all adolescents would achieve both Ca intakes ≥ 1050 mg Ca/day and sufficient vitamin D status ("ideal", shown in green). However, only 24.7% of teens met these criteria. The clinical relevance of the interaction we identified lies in the implication that significant improvements in fetal and neonatal bone outcomes can be achieved when adolescents move from the "inadequate" red area to either the "A" ***or*** "B" location; ie by achieving adequacy in just one of the two nutrients.

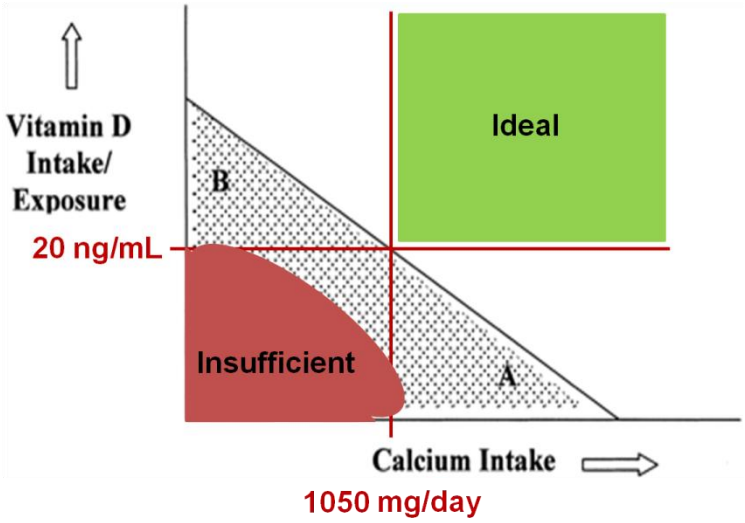

Figure 3.6 Conceptual Diagram of the Interaction between Maternal Ca Intakes ≥ 1050 mg/day and 25(OH)D > 20 ng/mL on Fetal and Neonatal Bone Outcomes in Pregnant Adolescents
In this cohort of pregnant adolescents, the interaction identified between maternal Ca intakes ≥ 1050 mg/day and achievement of 25(OH)D > 20 ng/mL implied a compensation between the two nutrients. Significant improvements in fetal femur and humerus Z-scores and birth length were observed when adolescents moved from the red "Insufficient" area to either the "A" or the "B" area. Furthermore, no differences in fetal femur or humerus Z-scores or birth length were observed between adolescents in the "A" or "B" compared to adolescents in the "Ideal" location. Adapted from DeLucia et al (33).

Sufficient 25(OH)D concentrations may be necessary to support renal and extra-renal production of $1,25(OH)_2D$ which is known to regulate efficiency of intestinal Ca absorption (34). Additionally, increased systemic concentrations or local production of $1,25(OH)_2D$ in the placenta may result in increased placental flux of Ca to the fetus (35;36). The clinical relevance of the observed increase in fetal long bone Z-scores with achievement of either 25(OH)D > 20 ng/mL or Ca intake ≥ 1050 mg/day is underscored by the number of teens (37%) that failed to meet either criteria.

In our adolescent population, the observed differences in long bone growth *in-utero* were associated with similar differences in birth length as a function of Ca intake. In the generated models of neonatal birth length, increasing maternal Ca to ≥ 1050 mg Ca/day was associated with a 0.54 cm increase in birth length. Data from Ca replete, skeletally mature populations may differ from that observed in adolescents, and prior data linking maternal Ca intake with infant birth length has been inconsistent. A study of 222 Caucasian women (>30 y of age, consuming average intakes of ~1300 mg Ca/day) found no relationship between that maternal Ca intake during pregnancy and infant birth length (37) but a randomized control trial in 256 healthy adult pregnant women showed that providing 2 g daily Ca supplementation from < 22 weeks gestation to term resulted in a non-significant 0.5 cm increase in birth length (11). These studies' results may have been impacted by a limited potential to benefit since they were undertaken in relatively well-nourished adult women.

In these adolescents the effect of maternal Ca intake on birth length was mediated by maternal vitamin D status, and only remained significant when maternal 25(OH)D was insufficient (≤ 20 ng/mL). A study of women in Iran reported that infants born to women who consumed adequate amounts of both Ca (> 1000 mg/day) and vitamin D (> 200 IU/day) were significantly longer at birth by 0.87 cm (38), compared to the 0.4 cm longer birth length we detected in our "Ca/D sufficient" pregnant adolescents. In our study, birth length was measured by clinical staff upon delivery using standard hospital practices which likely increased measurement error and variability. Our lack of standardization of neonatal birth length measures likely increased variability and only diluted our ability to detect significant associations.

A strength of our study design was the biochemical assessment of vitamin D status combined with dietary assessment of Ca intake, as no analogous biomarker for Ca exists. In this

population, vitamin D intake only explained 6% of the variation in 25(OH)D, emphasizing the need to use a biomarker for vitamin D status. As expected, dietary intake of vitamin D and Ca intake in this group were highly correlated, which necessitates that effects attributed to Ca intake be interpreted with caution. The collinearity of Ca and vitamin D intake is likely due to consumption of dairy/vitamin D fortified foods, and thus counseling pregnant adolescents to increase their consumption of Ca and vitamin D rich foods would be appropriate to ensure the gains in fetal skeletal outcomes attributed to Ca in these models. The wide range in Ca intakes and 25(OH)D concentrations observed, and the skeletal immaturity of this age group may have increased our ability to detect the associations noted. We assessed the impact of maternal nutritional status on fetal long bones in both the upper and lower limb. Presumably, both these bones are subject to the same regulatory processes *in-utero* (39), yet possible racial differences in these bone lengths may not be analogous as fetal femur, but not humerus lengths, may differ by maternal race (40;41). A similar trend for racial differences in long bone length approached significance in our data. At present, fetal biometry standard curves are not race-specific, but maternal race was controlled for in our models of fetal bone length to account for potential differences. It is noteworthy that, although physiological differences between the femur and humerus may exist, the nature of the interactions observed, and size of the effect of maternal Ca intake and vitamin D status on each of these two bones was similar.

The significant interaction between maternal Ca intake and 25(OH)D status that we observed in these teens suggests that consuming either an adequate Ca intake or achieving sufficient 25(OH)D status may partially attenuate the deficits in fetal long bone growth and neonatal birth length observed in pregnant adolescents who were Ca/D insufficient. Whether or not the findings in this group of adolescents are generalizable to skeletally mature women

remains unknown, yet the possibility merits investigation as nearly 50% of American women of child-bearing age consume < 1050 mg Ca/day (21), and vitamin D insufficiency is prevalent. These findings underscore the need for pregnant adolescents to ingest recommended Ca intakes and maintain optimal vitamin D status throughout gestation to ensure optimal fetal skeletal growth.

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FW, ZH, and KO designed research; BE, AM, BC, TM, and KO conducted the research; BE analyzed the data; BE and KO wrote the paper; KO has primary responsibility for final content. All authors read and approved the final manuscript. All authors have nothing to disclose.

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CHAPTER 4

PLACENTAL VITAMIN D RECEPTOR (VDR) EXPRESSION IS RELATED TO NEONATAL VITAMIN D STATUS AND FETAL BONE LENGTH IN PREGNANT ADOLESCENTS

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ABSTRACT

Vitamin D status may impact placental gene expression. To assess this possibility, relationships among placental vitamin D receptor (VDR) expression, fetal bone length, maternal Ca intake and maternal and neonatal vitamin D status were examined. The study was undertaken in 94 pregnant adolescents (≤ 18 y of age) who delivered at 39.9 ± 1.2 weeks of gestation. Serum 25(OH)D, 1,25$(OH)_2$D, and PTH were measured in maternal circulation at delivery and in cord blood obtained at birth. Maternal Ca intake was assessed via 24-hour recall. Fetal femur length Z-scores were generated from sonogram measures obtained at 34.3 ± 3.2 weeks gestation. Placental VDR expression (via Western blot) was significantly and inversely associated with neonatal 25(OH)D ($p = 0.012$); and a similar but non-significant trend was evident between placental VDR and maternal 25(OH)D ($p = 0.080$). The hormonal form of vitamin D in the neonate, 1,25$(OH)_2$D, was highly significantly positively associated with placental VDR expression ($p = 0.006$). Neonatal 1,25$(OH)_2$D and 25(OH)D concentrations were not significantly related. Adolescents with high placental VDR expression had a significantly more positive fetal femur length Z-score at late gestation ($p = 0.029$). Placental VDR expression remained a significant predictor of fetal femur length Z-score ($p = 0.018$) after controlling for maternal smoking, race, weight gain, height, Ca intake, 25(OH)D status, and placental weight. Insufficient neonatal 25(OH)D status and high neonatal 1,25$(OH)_2$D concentrations may impact VDR expression in the placenta to impact skeletal growth *in-utero*. The effects of placental VDR expression on placental function and transport of nutrients to the fetus warrant further investigation.

INTRODUCTION

All nutrients necessary for fetal growth and development are transported across the placenta over only 266 days in the human. Increasing attention has been focused on the placenta's ability to respond to signals of fetal demand and optimize transport of nutrients to the fetus within the context of maternal supply (1;2). The mechanisms and proteins involved in the regulation of placental nutrient transport is an evolving area of research.

Maternal vitamin D status, as measured by circulating 25(OH)D concentrations, has been linked to pregnancy outcomes and offspring risk of chronic disease (3). Increasing data have also found significant associations between maternal vitamin D status and both placental function and fetal growth (4). Low 25(OH)D has been linked with impaired fetal femoral development (5), reduced bone mineral content (BMC) and bone density in the neonate at birth (6;7), and persistent reductions in BMC in offspring at 9 years of age (8). The mechanisms responsible for these associations remain unknown.

Vitamin D-mediated regulation of gene expression at the level of the placenta may influence transport of Ca and other essential nutrients to the fetus. The 1α–hydroxylase enzyme (1α-OHase) is expressed in the kidney and in the placenta (9) and converts 25(OH)D into the hormonal form of vitamin D, 1,25$(OH)_2$D. This hormone binds to the vitamin D receptor (VDR) and regulates expression of genes containing vitamin D response elements (VDRE). The presence of both the 1α-OHase and the VDR in the placenta suggests that this organ has the capacity for endocrine as well as local paracrine and autocrine vitamin D activity (9;10).

There are limited data investigating the determinants and effects of VDR expression in the placenta, but other studies have established that VDR likely plays an important role in placental function. The VDR gene is completely unmethylated in the placenta (11). This lack of

epigenetic inhibition of transcription suggests that the placenta as an organ is "primed" for optimal vitamin D activity. These data, suggest that VDR likely plays a role in the modulation placental gene expression and as over 150 genes have been found to contain VDRE's (12), modulation of VDR expression in the placenta has the potential for significant downstream impact on placental function.

In-vitro studies using human cell lines have found that placental VDR expression is regulated by vitamin D metabolites (13). Data from animal models suggest that VDR plays a role in fetal growth and that this effect is mediated by maternal Ca intake (14) but in-vivo human data are lacking. To address this issue, we explored determinants of placental VDR expression in relation to maternal and neonatal vitamin D status in a group of pregnant adolescents. Possible associations between placental VDR and *in-utero* measures of fetal bone length were explored.

MATERIALS AND METHODS

Study Participants:

Placental tissue from 94 adolescents was obtained at birth. These 94 adolescents were part of a larger cohort (n = 157) of pregnant adolescents enrolled in a prospective study designed to assess maternal and fetal bone health and vitamin D homeostasis across gestation. All adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) clinic in Rochester, NY. Healthy pregnant adolescents were eligible to participate if they were ≤ 18 y of age, between 12 and 30 weeks gestation at entry into the study, were otherwise healthy, and were carrying a single fetus. Adolescents with known medical complications (such as HIV infection, diabetes, gestational hypertension, diagnosed eating disorders or malabsorption diseases) were ineligible to participate. At entry into the study data on race, ethnicity, pre-pregnancy weight, and smoking history were self-reported. Informed written consent was obtained from all participants, and the study was approved by the Institutional Review Boards of the University of Rochester and Cornell University. Data from this study population detailing vitamin D status (Chapter 2), bone turnover markers, serum and placental osteoprotegerin (Appendix 11) and fetal long bone growth (Chapter 3) are currently under review.

Up to three times during pregnancy, maternal dietary intake was assessed by 24-hour dietary recall and fetal biometry measures were obtained by sonogram. Fetal femur length Z-scores were generated using previously published equations derived from a large cohort (n = 929) of pregnant African American adolescents in Baltimore, MD, USA (15). At birth, infant weight and length were recorded by clinical staff using standard procedures. Sex and gestational age-specific infant birth weight Z-scores were generated from published equations (16). Immediately after delivery, the placenta was collected and weighed to the nearest 0.1 g.

Biochemical Analyses:

At delivery, a maternal and cord blood sample (10 mL) was obtained. Serum was separated from all blood samples and aliquots were immediately sent to a Quest Laboratory (participating in DEQAS) for assessment of 25(OH)D using the Diasorin RIA (Diasorin Inc, Stillwater, MN). According to the 2010 DRI, vitamin D insufficiency was defined as 25(OH)D $\leq$ 20 ng/mL (17). Intact parathyroid hormone (PTH) was analyzed in maternal samples using a commercially available ELISA (DSL Laboratories, Webster, TX). Calcitriol ($1,25(OH)_2D$) was analyzed in 60 of the 94 maternal samples and 51 of the 94 neonatal samples at Boston University in the lab of Dr. Michael Holick (Boston, MA), as previously described (18). Missing calcitriol data was due to insufficient serum volume for this analysis.

Placental Sample collection:

Placentas were processed shortly after delivery as previously described (19;20;Appendix 11). Placentas were weighed to the nearest 0.1 g and dimensions and shape recorded. The sac was trimmed, and five tissue sections (approximately one-square-inch each) were collected from representative sites. The maternal and fetal membranes were removed from each section. Samples were then pooled, homogenized, and frozen in small aliquots at -80^0C until analysis. Protein lysates were generated by homogenizing thawed placental tissue using a Polytron PT3100 tissue homogenizer (Kinematica, Lucerne, Switzerland) at 5,000 rpm in a hypertonic lysis buffer containing protease inhibitor. Homogenized tissue was centrifuged at 13,000 rpm for 20 minutes at 4^0C. Protein concentration in the supernatant was determined with the Bio-Rad assay (Bio-Rad, Hercules, CA). Lysates were diluted in SDS sample buffer and stored at -80^0C until analyses.

Western Blot:

Relative placental expression of VDR was determined by Western blot. Lysates were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked in Odyssey Blocking Buffer (Li-Cor BioSciences, Lincoln, NB) for one hour at room temperature and then probed with anti-human VDR, raised in rat (ab8756:AbCam, Cambridge, MA) at a dilution of 2µg/mL and anti-human β-actin, raised in rabbit (AbCam, Cambridge, MA) at 10.1 ng/mL overnight at 4^0C. Fluorescent secondary antibodies were used (Li-Cor BioSciences): anti-rat 800, raised in goat at a concentration of 0.2 µg/mL, and anti-rabbit 680, raised in goat at 0.1 µg/mL. All antibodies were made up in a solution of Odyssey blocking buffer diluted 1:1 in a phospho-buffered saline (PBS) solution. All antibodies were tested for cross-reactivity before collection of data. The VDR and β-actin bands were quantified using the Odyssey infrared imaging system (Li-Cor Biosciences), and the ratio of VDR to β-actin calculated. HeLa nuclear lysate (ab14655:AbCam, Cambridge, MA) was used as a positive control for VDR. Thirteen placental samples and a standard control placental sample were run on every gel. This standard control placenta sample originated from a single lysate that was generated from an adult placenta (from an individual not participating in this study) collected at term, as previously described. If the VDR: β-actin ratio of the control placenta sample on any given gel was over two SD from the mean observed for this control sample on all gels run, data from that gel was not used, and all study placentas on that gel were re-analyzed. Thus, the control placental samples' VDR: β-actin ratios from every gel were normally distributed and all within two SD from the mean. Intra-membrane variation was controlled for by normalizing the VDR: β-actin ratio of each study sample to the standard control placental sample that was run on that gel.

Statistical Methods:

Analyses were performed using SAS 9.2 and JMP 8.0 (SAS Institute Inc, Cary, NC). Results are reported as the mean ± standard deviation (SD) unless otherwise stated. Independent t-tests or ANOVA were used to determine if normally distributed variables differed by categories of vitamin D status or placental VDR expression, and the Wilcoxon Rank sum test was utilized for nonparametric data. Simple linear regression was used to assess relationships between 25(OH)D or $1,25(OH)_2D$ and placental VDR expression, and between placental VDR expression and fetal femur Z-scores. Multiple linear regression was used to model the relationship between placental VDR expression and fetal femur Z-scores, while controlling for covariates including placental weight (to control for placental size). An interaction term between placental VDR expression and placental weight was included in all models but was not significant in any model, and thus will not be discussed herein. Normality was tested using the Shapiro-Wilks test, and non-normally distributed variables were log transformed as necessary to ensure normality of the residuals. P values < 0.05 were considered significant, and P values between 0.05 and 0.10 were considered trends.

RESULTS

Subject Characteristics

Characteristics of the 94 adolescents and their neonates are presented below in **Table 4.1**.

Table 4.1. Characteristics of 94 Pregnant Adolescents from whom Placental Tissue was Obtained, and their Neonates at Birth

Characteristic	Mean ± SD (n)
Maternal Age Enrollment (y)	17.1 ± 1.1 (94)
African American Caucasian	62.8% (59) 37.2% (35)
Hispanic Non-Hispanic	26.6% (25)[1] 73.4% (69)
Parity ≥ 1	10.6% (10)
Pre-pregnancy BMI (kg/m^2)	25.1 ± 5.9 (93)
Weight Gain (Lbs)	39.5 ± 17.7 (93)
Ca Intake (mg/d)	904 ± 358 (93)
Infant Sex (Male)	51.1% (48)
Birth Weight (g)	3287 ± 472 (94)
Birth Weight Z-Score	-0.382 ± 0.890 (94)
Placental Weight (g)	601.4 ± 121.7 (91)
Fetal Femur Length Z-Score[2]	-0.606 ± 0.934 (94)
Maternal 25(OH)D at delivery (ng/mL)	21.5 ± 12.9 (91)
Maternal $1,25(OH)_2D$ at delivery (pg/mL)	104.2 ± 33.3 (60)
Neonatal 25(OH)D (ng/mL)	21.2 ± 10.7 (89)
Neonatal $1,25(OH)_2D$ (pg/mL)	47.9 ± 17.7 (51)

[1] 60.0% of Caucasians self-identified as Hispanic vs. 6.8% of African Americans, $p<0.0001$.
[2] Measured at 34.3 ± 3.2 weeks gestation

Maternal age ranged from 13.6 to 18.7 years. Approximately 38% of adolescents were overweight (BMI $\geq$ 25.0 kg/m^2) pre-pregnancy, with pre-pregnancy BMI ranging from 15.0 to 42.1 kg/m^2. One adolescent reported her race as American Indian. Study results did not differ if this adolescent was excluded or grouped with either the Caucasian or African American cohorts. In order to collapse maternal race into a bivariate variable, the American Indian adolescent was grouped with the African American cohort. A larger percentage of Caucasians reported their ethnicity as Hispanic (60.0%) than did African American participants (6.8%; $p < 0.0001$). Approximately 12% of adolescents were currently smoking during pregnancy (an average of 0.5 packs of cigarettes/day).

Fetal femur length measures were obtained in the third trimester of pregnancy at week 34.3 ± 3.2 of gestation. Fetal femur Z-scores were highly variable ranging from -2.70 to 2.20. All but one of the 94 adolescents delivered at term (39.9 ± 1.2 weeks); one infant was premature (36.9 weeks gestation). This is an underestimate of the rate of preterm delivery normally reported in this age group and evident in the larger cohort, and is due to the decreased likelihood of obtaining placental tissue from a preterm delivery. Birth weight Z-scores were variable, ranging from -2.58 to 2.09. As evident in the larger cohort (Chapter 2), neonates born to Caucasian adolescents had significantly higher 25(OH)D concentrations at birth when compared to neonates born to African American adolescents ($p = 0.004$). Neonatal 25(OH)D was highly correlated with maternal 25(OH)D ($p < 0.0001$, $n = 86$, $R^2 = 0.63$). Neonatal circulating $1,25(OH)_2D$ was not correlated with 25(OH)D in the mother or the neonate.

Maternal Determinants of Placental VDR Expression

VDR was detected in all placental homogenates analyzed (**Figure 4.1)**. Placental VDR was not significantly related to maternal 25(OH)D, although a negative association approached

significance ($p = 0.080$, $R^2 = 0.03$, $n = 91$). Placental VDR was not significantly related to maternal $1,25(OH)_2D$ or PTH. VDR expression was not related to maternal pre-pregnancy BMI, weight gain, gestational age, placental weight, smoking status, Ca intake, or maternal race or ethnicity.

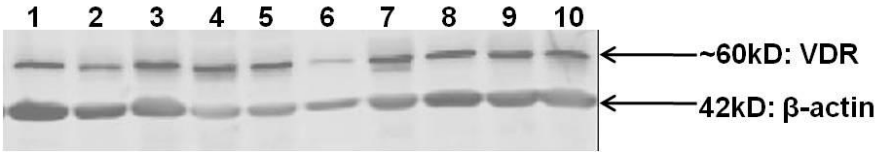

Figure 4.1 VDR Expression is Detectable via Western Blot in all Placental Tissue Collected from 94 Pregnant Adolescents
VDR expression was assessed via Western blot in protein lysates generated from placental tissue obtained at delivery from 94 pregnant adolescents (age 17.1 ± 1.1 years) at delivery. Shown is a representative blot with individual placental lysate samples in lanes 1 - 10.

Neonatal Determinants of Placental VDR Expression

Placental VDR was significantly negatively associated with neonatal 25(OH)D ($P = 0.012$, $n = 89$, $R^2 = 0.06$; **Figure 4.2A**), and significantly positively associated with neonatal $1,25(OH)_2D$ ($P = 0.006$, $n = 54$, $R^2 = 0.14$; **Figure 4.2B**). Placental VDR did not differ by neonatal race or ethnicity or infant sex.

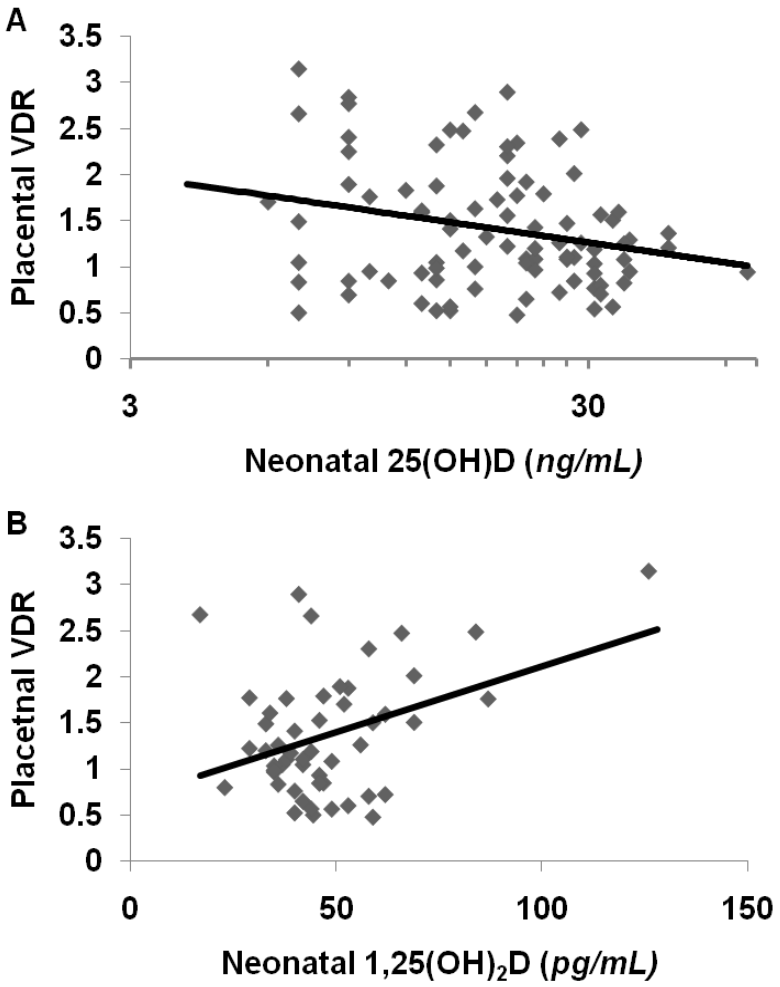

Figure 4.2 Placental VDR Expression is Negatively Associated with Neonatal 25(OH)D and is Positively Associated with Neonatal $1,25(OH)_2D$
A: Relative placental VDR expression via western blot (a unit-less measure) was inversely related to Ln (neonatal 25(OH)D concentrations) ($p = 0.018$, $R^2 = 0.06$, $n = 89$). B: Placental VDR expression was positively related to neonatal $1,25(OH)_2D$ concentrations ($p = 0.006$, $R^2 = 0.14$, $n = 51$).

Figure 4.3 below summarizes the inter-relationships between maternal and neonatal vitamin D status and placental VDR expression.

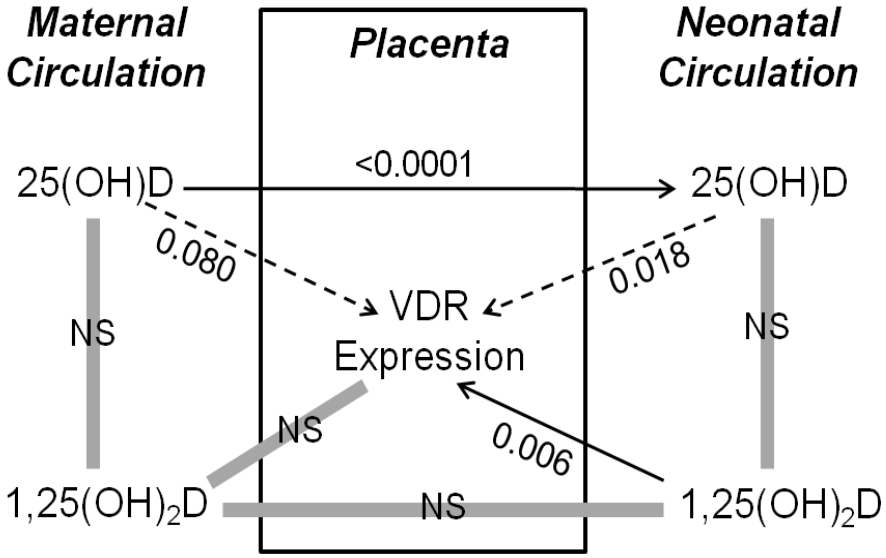

Figure 4.3. Summary of Inter-Relationships Between Maternal and Neonatal Vitamin D Status and Placental VDR Expression
Maternal and neonatal determinants of placental VDR expression were explored. Shown are the inter-relationships and corresponding P values between maternal and neonatal vitamin D status and placental VDR expression. Solid black lines represent significant positive relationships. Dashed lines represent significant inverse relationships. Gray lines indicate variables are not significantly (NS) associated. Neonatal calcitriol was the most significant predictor of placental VDR expression.

Relationships between Placental VDR Expression and Neonatal Outcomes

Placental VDR was not linearly related to neonatal birth weight, birth weight Z-score, or fetal femur length Z-score at 34.3 ± 3.2 weeks. However, when placental VDR expression was categorized as either above and below the median expression observed, adolescents with placental VDR expression above the median had significantly longer fetal femur lengths (higher femur length Z-score) than those with placental VDR expression below the median value (P = 0.029, n = 94) (**Figure 4.4**). The difference in mean femur length Z-score between those with placental VDR above and below the median expression amounted to 0.420 SD. At 34.3 weeks

gestation, a 0.420 difference in femur length Z-score is equivalent to a 1.15 mm difference in femur length, a value that represents 1.7% of total bone length at this stage of development.

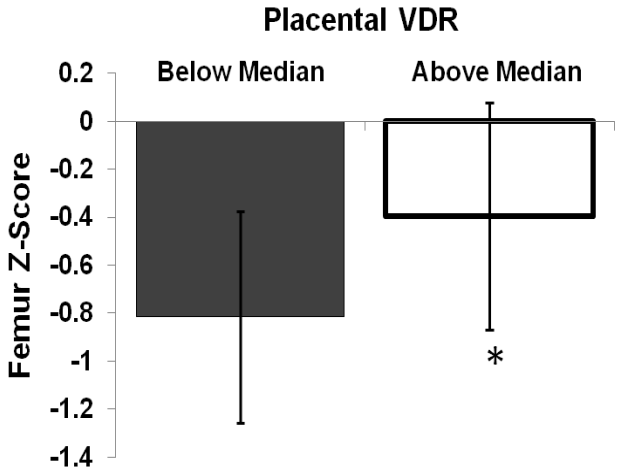

Figure 4.4 Fetal Femur Z-Score is Higher in Adolescents with Placental VDR Expression Above verses Below the Median Expression Observed
Fetal biometry was assessed at 34.3 ± 3.2 weeks gestation and age-specific fetal femur Z-score generated using published equations (15). Placental vitamin D receptor (VDR) expression was assessed by Western blot (n = 91). Fetal femur Z-scores were significantly more positive by 0.420 SD in adolescents with VDR expression above the median when compared to Z-scores evident in those with placental VDR expression below the median (*p = 0.030, n = 91).

After controlling for maternal smoking, race, weight gain, height, Ca intake, 25(OH)D sufficiency, and placental weight, placental VDR expression remained a significant predictor of fetal femur length Z-score (parameter estimate = 0.018, n = 90) (**Figure 4.5)**. Controlling for 25(OH)D sufficiency as opposed to 25(OH)D as a linear variable improved the model's predictability. Adding placental VDR expression to the model of fetal femur Z-score, increased the R^2 from 0.17 to 0.21. Adjusted fetal femur Z-score was 0.409 SD higher in placentas with VDR expression above the median than in those below the median (p = 0.020).

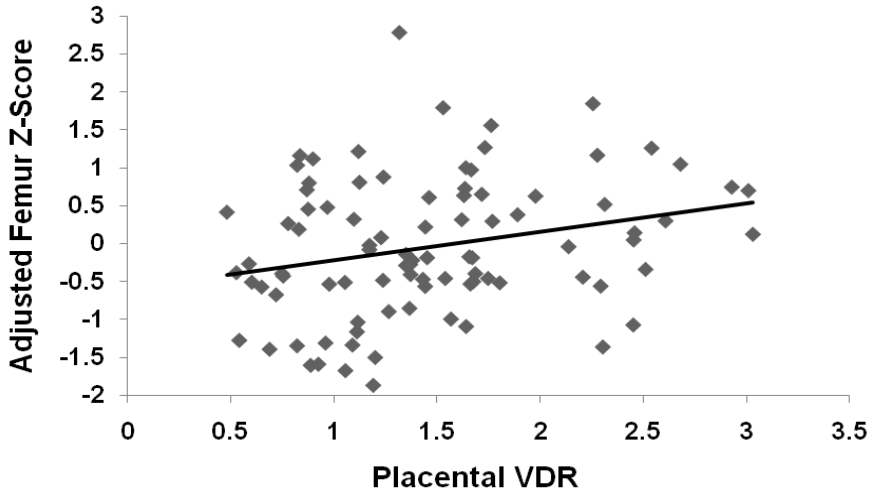

Figure 4.5 Placental VDR Expression is Positively Associated with Adjusted Fetal Femur Z-Score

Fetal biometry femur length Z-scores were generated using published equations (15). In a multivariate model controlling for significant covariates, placental vitamin D receptor (VDR) expression (via western blot) was significantly positively associated with adjusted femur length Z-score ($p = 0.018$, $R^2 = 0.06$, $n = 90$).

DISCUSSION

In this cohort of racially diverse pregnant adolescents, a significant positive relationship between neonatal 1,25$(OH)_2$D and placental VDR expression was observed. Placental VDR expression was also significantly negatively associated with neonatal 25(OH)D. The significant differences in VDR expression were associated with a biologically relevant outcome as evidenced by the significant relationship between VDR expression and fetal femur length in-vivo.

Newborns with low vitamin D status (25(OH)D) had significantly higher placental VDR expression. To our knowledge this association has not previously been reported in human or animal studies. In these adolescents, the negative association between maternal 25(OH)D and placental VDR did not reach significance, even though the fetus is completely dependent on maternal supply of 25(OH)D *in-utero*, and neonatal 25(OH)D concentrations are highly correlated with 25(OH)D status (21;22). Whereas maternal and neonatal 25(OH)D were significantly correlated in these adolescents, neonatal 25(OH)D explained twice as much variation in VDR expression compared to maternal 25(OH)D. The mechanism for this association is unknown, but VDR expression may be increased as a compensatory response to low neonatal 25(OH)D status, which may limit substrate availability for local placental 1,25$(OH)_2$D production.

Of the variables measured, the strongest determinant of placental VDR expression was neonatal 1,25$(OH)_2$D concentration. Unlike the negative association seen for 25(OH)D, higher concentrations of neonatal 1,25$(OH)_2$D were associated with greater placental VDR expression. The concentration of this hormone in the neonate explained twice as much variability in VDR expression compared to concentrations of its precursor (25(OH)D). Furthermore, if both

25(OH)D and 1,25$(OH)_2$D were entered into a model together, 25(OH)D was no longer significantly associated with VDR expression. Of interest, the significant relationship between 1,25$(OH)_2$D and VDR expression was evident only in the neonate; maternal 1,25$(OH)_2$D concentrations at delivery were unrelated to VDR expression. In this cohort and other studies of pregnant adult women there is a lack of association between neonatal and maternal 1,25$(OH)_2$D concentrations at delivery (23;24). This is because, unlike 25(OH)D, the fetus is capable of synthesizing 1,25$(OH)_2$D; animal models show that the 1α-hydroxylase is active in the embryonic state, and fetal renal 1α-hydroxylase near term is similar to maternal renal activity and significantly higher than non-pregnant adults (25;26). Studies in lambs have been used to demonstrate that fetally derived 1,25$(OH)_2$D regulates fetal Ca homeostasis independently of maternal 1,25$(OH)_2$D (27). The findings from our study in adolescents are significant because they indicate that, fetal and not maternal 1,25$(OH)_2$D serves as a signal to regulate placental VDR expression. Calcitriol is a positive regulator of VDR expression in mammalian cells in-vitro (28). Analogous regulation has been established in human placental choriocarcinoma cell lines (13), confirming a plausible mechanism for the association detected.

Increased VDR expression may result in increased placental capacity for Ca transport, as several genes that play significant roles in placental Ca transport are up-regulated by 1,25$(OH)_2$D,. These include the Ca import protein TRPV6 (29;30), and the Ca-chaperone proteins, calbindin D9K and D28K (13;31;32). Furthermore, 1,25$(OH)_2$D treatment of the placental syncytiotrophoblast cell line (JEG-3) leads to a dose-dependent increase in Ca uptake (33). Although this syncytiotrophoblast model was initially derived from a choriocarcinoma cell line and may undergo different genetic regulation than healthy placental cells in-vivo, these data support the idea that increased 1,25$(OH)_2$D / VDR mediated activity may increase placental Ca

flux. It is possible that increased neonatal $1,25(OH)_2D$ may serve as a signal to increase VDR expression in the placenta, to increase placental Ca supply to the fetus. A limitation of our study was that we did not relate VDR expression with expression or activity of other genes known to be impacted by this receptor.

If increased VDR expression impacts placental Ca transfer *in-utero*, this may influence Ca accrual in the fetus. In this cohort, adolescents with higher VDR expression in the placenta had more positive fetal femur Z-scores. To our knowledge, this is the first time VDR expression in the placenta has been linked with a functional outcome of neonatal skeletal development. We have previously reported on determinants of fetal femur Z-score in the larger adolescent population (Chapter 3). It is of note that adding VDR to the covariates previously identified increases the explained variability in femur length Z-score by 4%. A limitation of this study is the lack of bone mineral content/density assessment in the neonate.

The critical role that VDR plays in skeletal development is evident by insufficient mineralization and short adult stature observed in humans with non-functional vitamin D receptor (vitamin D dependent rickets type II, VDDR-II) (34). Mice with a knockout in the VDR exhibit the hallmarks of human VDDR-II (35), and allow for further characterization of phenotype *in-utero* and during pregnancy. Both homozygous and heterozygous VDR knock out pups are smaller at birth, exhibit defective skeletal mineralization and elevated concentrations of $1,25(OH)_2D$ (14;36), supporting the idea that VDR contributes to optimization of bone development *in-utero*.

However, the role that VDR plays in placental Ca transfer and fetal skeletal mineralization in mice may be redundant, as the characteristic impaired bone formation, hypocalcemia and failure to thrive in VDR knockout animals do not become fully evident until

weaning (37), and VDR knockout phenotypes in the fetus/newborn pup can be rescued if pregnant dams are fed a high-Ca diet (14). The ability to compare murine data to humans is limited by species and dietary differences. We and others have documented that pregnant adolescents often ingest suboptimal Ca intakes in the context of suboptimal vitamin D status (Chapter 2 & 3(38). These current VDR findings suggest that placental VDR expression in teen pregnancy may be responsive to circulating $1,25(OH)_2D$ in the neonate and facilitate increased fetal Ca supply. This mechanism may become particularly relevant in human pregnancy in the context of maternal skeletal immaturity combined with inadequate maternal Ca intake and/or 25(OH)D concentrations. In such a case, such relationships may be undetectable in whole genome knockouts and well-nourished animals.

Epidemiologic studies also suggest that the VDR plays a role in fetal growth by demonstrating that fetal (and placental) VDR genotype modifies the association between maternal 25(OH)D status and infant birth size (39). Maternal D deficiency has been linked to reduced infant birth weight only in infants with two most functionally active *FokI* polymorphisms (FF or Ff VDR genotype) (39). Placental VDR expression has also been associated with more complex pregnancy complications such as pre-term delivery; placentas collected from preterm births exhibit lower VDR expression than placentas from normal, uncomplicated deliveries (40). Future research is warranted to understand the totality of how VDR expression impacts fetal outcomes.

This study is the first to correlate placental VDR expression in the human placenta with both neonatal calcitropic hormones and with a fetal measure of bone acquisition. Higher placental VDR expression was evident among neonates with higher $1,25(OH)_2D$ concentrations which indicates that placental VDR expression may be "responsive" to fetal $1,25(OH)_2D$

production. We have also shown that placental VDR expression is associated with a measure of fetal bone accrual – fetal femur length Z-score. These results increase our understanding of the "responsive" nature of the placenta, but more research is needed to elucidate the mechanisms by which VDR expression impacts placental Ca transfer. And as ~500 genes are in some way regulated by VDR (41) research is warranted to fully detail the impact and role of VDR on placental nutrient transport, and fetal growth.

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CHAPTER 5

IN-VIVO MEASURES OF MATERNAL-FETAL CALCIUM TRANSPORT ARE ASSOCIATED WITH PLACENTAL VDR EXPRESSION AND FETAL LONG BONE LENGTH IN PREGNANT ADOLESCENTS*

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ABSTRACT

Little is known about how placental Ca transport is regulated *in-utero*, and in-vivo studies in humans are lacking. We utilized a dual stable Ca isotope approach to obtain in-vivo measures of maternal-to-fetal Ca transport in pregnant adolescents. Maternal Ca intake was assessed via 24-hour recall; and 25(OH)D, PTH, and $1,25(OH)_2D$ were assessed in adolescents at mid-gestation and delivery, and in cord blood at birth. Fetal femur and humerus length Z-scores were generated from sonogram data obtained in the third trimester. Placentas were collected upon delivery and vitamin D receptor (VDR) expression assessed via Western blot. We administered ^{44}Ca orally and ^{42}Ca intravenously to pregnant adolescents (n = 12) early in labor, and assessed enrichment in maternal circulation two hours post-dosing, and in cord blood at delivery. We generated quantitative measures of maternal-to-fetal Ca transport by calculating ratios of neonatal to maternal enrichment, controlling for time-to-delivery. Study variables that were significantly related to maternal-fetal Ca transfer were identified. Relationships between this transfer and fetal long bone Z-scores were explored. Maternal-fetal ^{42}Ca transfer did not differ from ^{44}Ca transfer and neither was associated with Ca intake, PTH, or $1,25(OH)_2D$. Maternal 25(OH)D > 20 ng/mL at mid-gestation and placental VDR expression were significantly positively associated with maternal-fetal ^{42}Ca transfer. Time-adjusted measures of maternal-fetal ^{42}Ca and ^{44}Ca transfer were significant in models of fetal femur and humerus Z-scores, respectively. The findings from this novel study design suggest that maternal vitamin D status and placental VDR play a role in the regulation of maternal-fetal Ca transfer, and that this Ca is incorporated into the developing fetal skeleton as reflected in measures of long bone length.

INTRODUCTION

Over the course of gestation ~30 g of calcium (Ca) is actively transferred across the placenta to the developing fetus (1). Placental Ca flux markedly increases in the third trimester, when ~80% of net placental Ca transfer occurs to support peak rates of fetal skeletal mineralization (2). Rates of fetal Ca deposition peak at 35 weeks gestation, and are thought to approximate 330 mg Ca/day (3). In the context of adolescent pregnancy, competition for nutrients may arise when fetal development occurs in an environment that is already physiologically stressed due to the additional Ca demands of adolescent growth.

In order to meet this additional Ca demand, maternal intestinal Ca absorption increases early in pregnancy and remains elevated until term (4). Biochemical markers of bone resorption and formation both increase significantly across gestation, suggesting that mobilization of maternal skeletal stores play a role in satisfying fetal Ca demand (5). When a woman becomes pregnant during her adolescent years, the Ca demands of the developing fetus are added to those of adolescent growth, and these normal pregnancy-induced physiological adaptations may not be adequate to meet the combined maternal and fetal Ca demand. Additionally, pregnant adolescents are likely to have a high prevalence of vitamin D insufficiency (5-8)Chapter 2) and to consume low quality diets that do not meet Ca requirements (9;10)Chapter 2). For all these reasons, it may be more difficult for pregnant adolescents to satisfy Ca demands than adult pregnant women.

Maternal vitamin D status and Ca and milk intake have been linked to a variety of fetal bone outcomes such as bone length, mineral content, and density (11-17), as well as bone mineral density of offspring later into childhood (18;19). We have recently shown that maternal Ca intake and vitamin D status interact to impact fetal femur and humerus length as well as

neonatal birth length in a pregnant adolescent population (Chapter 3). However, little is known about the impact of maternal Ca intake and vitamin D status on placental Ca transport, and the degree to which this transport is impacted by suboptimal intake or maternal nutrient status. To date, we have shown that 25(OH)D is associated with placental vitamin D receptor (VDR) expression, and that placental VDR expression is in turn associated with fetal femur length (Chapter 4). These findings suggest that local VDR expression and vitamin D activity at the level of the placenta may play a role in regulation of placental Ca flux, but at this time such mechanisms remain to be proven.

Placental Ca transfer is methodologically challenging to measure in humans, as radio-active approaches are unsafe during pregnancy. Stable Ca isotopes provide a safe and effective way of tracing homeostatic movements of Ca within the human body. In order to address the lack of data regarding *in-utero* Ca flux to the human fetus, we undertook a dual stable Ca isotope study, administering isotopes to pregnant adolescents early in labor, and then assessing Ca enrichment in maternal circulation post-dosing, and in cord blood collected at delivery. The purpose of this study was two-fold. First, we aimed to characterize neonatal enrichment, derive measures of placental Ca transfer and assess what variables were associated with these measures. Possible associations between maternal vitamin D and calcitropic hormone status and neonatal enrichment were also explored. Secondly, we sought to explore if measures of maternal-to-fetal Ca transfer at parturition were related to measures of neonatal skeletal size that were obtained *in-utero* and at birth.

MATERIALS AND METHODS

Study Participants

Twelve adolescents participated in this isotope study that took place at delivery. All 12 teens were enrolled in a larger prospective cohort study (n = 171) designed to characterize longitudinal changes in maternal and fetal bone health across gestation, as previously described (Chapter 2, 3, 4). Adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) clinic in Rochester, NY. All were between 16 and 18 years of age, primiparous, carrying a single fetus and were otherwise healthy. Exclusion criteria included known medical complications or diseases such as HIV infection, diabetes, pre-diagnosed gestational hypertension, diagnosed eating disorders, and malabsorption diseases. Informed written consent was obtained from all participants, and all procedures were approved by the Institutional Review Boards of the University of Rochester and Cornell University. The consent form for this study is presented in **Appendix 10**. Maternal race, ethnicity, pre-pregnancy weight, and smoking history were self-reported. Each adolescent was asked to attend up to three study visits across pregnancy. At each study visit maternal anthropometrics were recorded and a 24-hour dietary recall was administered by study personnel utilizing food models to help estimate portion sizes. All 24-hour recalls were analyzed by a registered dietician using the Nutrition Data System for Research (NDSR: University of Minnesota, Minneapolis, MN, versions 2006, 2008, and 2009) in the Clinical Research Center at the University of Rochester (Rochester, NY).

At each of the three visits, a fetal sonogram was performed, and fetal femur and humerus length were recorded by trained sonographers. Fetal femur length Z-scores were generated from previously published equations derived from a large cohort (n = 949) of pregnant adolescents in Baltimore, MD (20). Fetal humerus length Z-scores were calculated from the published curves

generated by Chitty et al. (21). The femur and humerus length Z-sores used in this study were generated using the last prenatal sonogram performed (at 34.2 ± 4.3 weeks). At birth, infant weight and length were recorded by clinical staff using standard procedures. Maternal blood volume at delivery was estimated assuming a blood volume of 70.0 ml/kg, (22) and neonatal blood volume was estimated from a formula published by Linderkamp et al. based on neonatal weight, length, hemoglobin, and hematocrit (23).

Placental Vitamin D Receptor (VDR) Expression

Placental tissue was collected within 30 minutes of delivery and placental protein lysates were generated as previously described (Chapter 4). Placental expression of VDR was determined via western blot as previously described (Chapter4).

Biochemical analyses

Maternal blood (10 mL) was obtained at mid-gestation (~28 weeks) and at delivery. At delivery, a 10 mL sample of cord blood was also collected. All blood samples were allowed to clot, then serum was separated and stored at -80^{0}C until analysis. In each of the serum samples collected, serum total Ca concentration was measured using a Modular (P) Chemistry Automated System (Roche Diagnostics, Indianapolis, IN) at the University of Rochester, and also using a Dimension Xpand Plus (Siemens Healthcare Diagnostics, Deerfield, IL) at Cornell University. As the values obtained did not statistically differ, the mean of both of these values was used. In each maternal and cord serum sample collected, 25-hydroxyvitamin D (25(OH)D) was measured by Quest Diagnostics Laboratories (Rochester, NY), using the Diasorin RIA (Diasorin Inc, Stillwater, MN). This laboratory participates in the Vitamin D External Quality Assessment Scheme (DEQAS) as a means of quality assurance. Vitamin D insufficiency was defined as 25(OH)D ≤ 20 ng/mL. Adolescents who were vitamin D insufficient at mid-gestation were

offered daily 400 IU vitamin D_3 supplements at their next prenatal appointment, to take daily over the remainder of gestation. Calcitriol ($1,25(OH)_2D$) was measured by Heartland Assays, Inc. (Ames, IA) using a competitive RIA procedure as previously published (24). Intact parathyroid hormone (PTH) was measured using a commercially available ELISA (Biomerica Inc, Newport Beach, CA). Elevated PTH was defined as PTH ≥ 46 pg/mL. Leptin was analyzed using a commercially available ELISA from Millipore (Billerica, MA).

Isotope Preparation & Administration

Isotopes were obtained from Oakridge National Laboratory (Oakridge, TN) as ^{42}Ca (87.7% enriched) and ^{44}Ca (96.4% enriched). The isotope solutions were prepared as isotonic solutions of Ca-chloride and certified for sterility and pyrogenicity by Anazao Health (Tampa, FL). Single use vials of ^{42}Ca for intravenous use contained 5.0 mL of an isotonic saline solution at 0.224 mg ^{42}Ca/mL, to provide ~1 mg ^{42}Ca per vial. Single use vials of ^{44}Ca containing 3.0 mL of an isotonic saline solution at 0.970 mg ^{44}Ca/mL each provided ~3 mg of ^{44}Ca per vial. The Ca isotopic composition of the final solutions was confirmed using a Thermo Scientific Triton TI magnetic sector thermal ionization mass spectrometer (TIMS; Thermo Fisher Scientific Inc, Bremen, Germany). The total Ca concentration of the vials was confirmed using atomic absorption spectrophotometry (AAS; Perkin Elmer Analyst 800, Perkin Elmer, Norwalk, CT).

When participants were admitted to the hospital at ≥ 37 weeks gestation, and were approximately 3 cm dilated (as assessed by medical staff), the oral and intravenous isotopes were administered. Isotopes were drawn up in a syringe, and weighed to the nearest 0.001 g in the Highland Hospital Pharmacy. The intravenous dose was administered through an in-dwelling heparin-lock over the course of five minutes. A 5 mL normal saline flush was administered through the heparin lock after dosing to ensure full delivery of all isotope. Immediately after the

^{42}Ca dose was administered, the ^{44}Ca was mixed in a small volume (~20 mL) of water flavored with drink mix (sugar, calorie, and vitamin free; 4C Food Corp., Brooklyn, NY) and administered to the adolescent. Two ~20 mL rinses of the cup were also consumed by the participant to ensure all isotope was ingested. The empty oral and intravenous dosing syringes were post-weighed on the same scale as was used for the pre-dosing weight, to obtain an exact measure of the isotope dose delivered. Two hours after the intravenous dosing, a 10 mL venous blood sample was obtained from the participant, and cord blood was collected at delivery for isotopic analysis. Blood samples were allowed to clot before serum was removed and frozen at -80^{0}C until analyses.

Determination of Isotope Content & Ca Absorption

Calcium was precipitated from serum using ammonium oxalate, as previously described (25). Precipitated Ca was re-suspended in 3% Ultrex Nitric Acid and ~10 uL was loaded onto a rhenium filament. Calcium isotopic ratios were measured by TIMS (Thermo Scientific Triton TI, Thermo Fisher Scientific Inc, Bremen, Germany). The ratio of each administered isotope to ^{43}Ca was measured and the degree to which each ratio was increased over the natural abundance ratio (NA) was calculated as delta-percent excess (Δ-% excess) as follows:

$$\textbf{Δ-\% excess } {}^{\mathbf{42}}\mathbf{Ca} = \frac{{}^{42/43}\text{Ca} - \text{NA } {}^{42/43}\text{Ca}}{\text{NA } {}^{42/43}\text{Ca}} \text{ X } 100 \qquad \text{(Eq. 1)}$$

The same equation was used to calculate Δ-% excess of ^{44}Ca in both maternal and cord blood samples. The NA ratios used were: $^{42/43}$Ca = 4.799290; $^{44/43}$Ca = 15.457060 and the relative standard deviations of natural abundance measures was 0.0135% and 0.0118%, respectively. The following abbreviations are used to represent enrichment of ^{42}Ca and ^{44}Ca detected in the maternal and cord blood samples:

^{42}**Ca** $_{(M)}$ = Δ-% excess ^{42}Ca detected in maternal blood at two hours post-dosing

^{44}**Ca** $_{(M)}$ = Δ-% excess ^{44}Ca detected in maternal blood at two hours post-dosing

^{42}**Ca** $_{(C)}$ = Δ-% excess ^{42}Ca detected in cord blood

^{44}**Ca** $_{(C)}$ = Δ-% excess ^{44}Ca detected in cord blood

During pregnancy, the in-vivo dynamics of Ca partitioning are complex. **Figure 5.1** diagrams the entrances and exits impacting the size of the miscible Ca pool in a pregnant woman. During pregnancy, this pool expands significantly (4) due to increases in rates of bone turnover, blood volume expansion and increased intestinal Ca absorption. The placental/fetal unit serves as an additional compartment to which Ca is transferred out of the miscible Ca pool. The rates of bone turnover and the size of the miscible Ca pool are also considerably higher on both a net mg and a mg/kg basis in children and neonates due to the high rates of skeletal Ca accretion occurring in these groups (26).

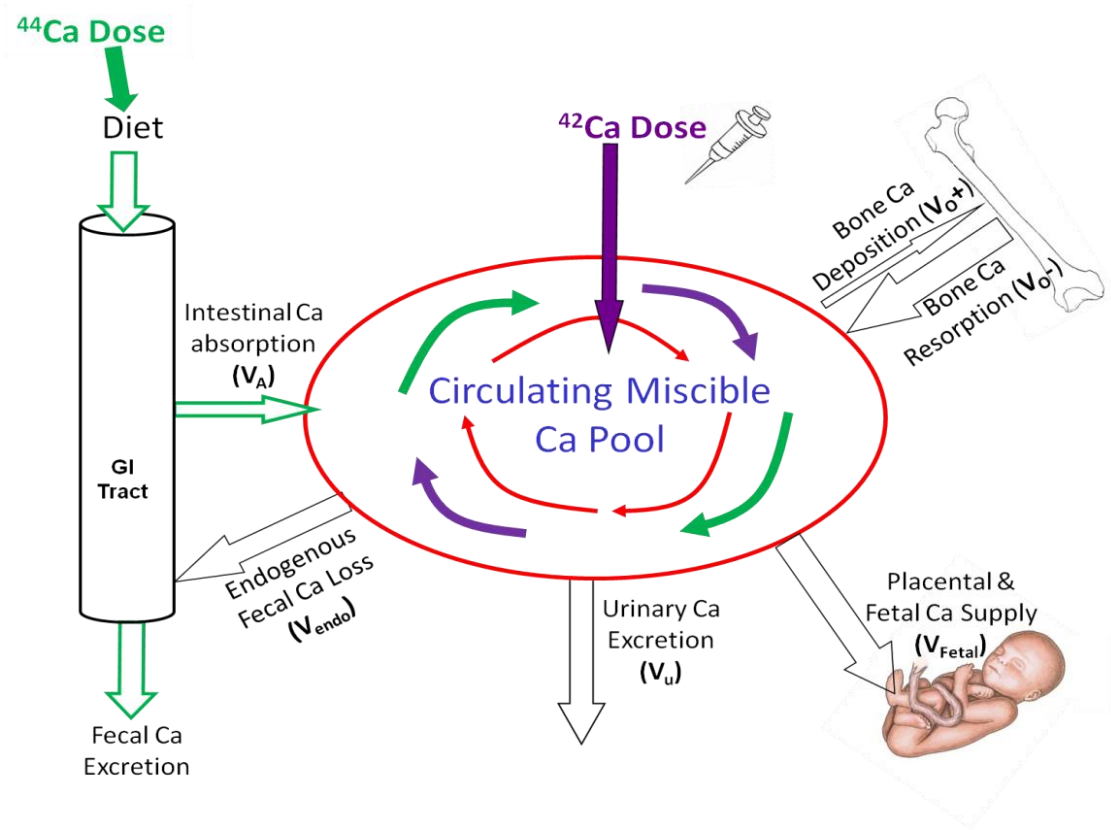

Figure 5.1 Model of Calcium Dynamics that Impact Calcium Balance During Pregnancy Rates of entry into and excretion from the miscible Ca pool are altered during pregnancy and vary on an individual basis. Calcium enters the miscible Ca pool from absorption of dietary Ca intake (V_A) and resorption from bone (V_O-). Calcium is lost to endogenous fecal losses (V_{endo}), renal losses in the urine (V_u), bone Ca deposition (V_O+), and Ca transfer to the fetoplacental unit (V_{Fetal}). In this study ^{44}Ca was administered orally (green arrow), and then assessed in maternal circulation and neonatal circulation (cord blood). Intravenous ^{42}Ca was administered directly into the miscible Ca pool, (purple arrow). Enrichment of ^{42}Ca in maternal circulation is a function of the size of the miscible Ca pool and rates of exit from this compartment. Enrichment of ^{42}Ca and ^{44}Ca in neonatal circulation will provide insight into the degree of Ca transferred from maternal to fetal circulation.

We are unable to measure neonatal usage of Ca tracer that is transferred to the fetus after maternal dosing. In general, studies in adult populations, neonates, and preterm infants have found that after intravenous administration of Ca isotope, Ca enrichment from the miscible Ca pool declines in an exponential manner (27). Because of the rapid Ca clearance rate observed,

we undertook this study in full-term pregnant adolescents admitted for delivery of their infant, in order to be able to determine isotopic enrichment of cord blood obtained within a matter of hours post maternal dosing. An additional challenge in assessing placental transfer of Ca to the fetus relates to variability in the time that elapses between administration of isotope to the pregnant teen and delivery of the neonate (and collection of cord blood). **Figure 5.2** shows the clearance of ^{42}Ca enrichment from serum after intravenous administration non-pregnant adolescent girls (28). Superimposed on this curve of ^{42}Ca decay are the time intervals in which we obtained the post-dosing maternal blood sample, and the wide time interval in which cord blood samples were obtained.

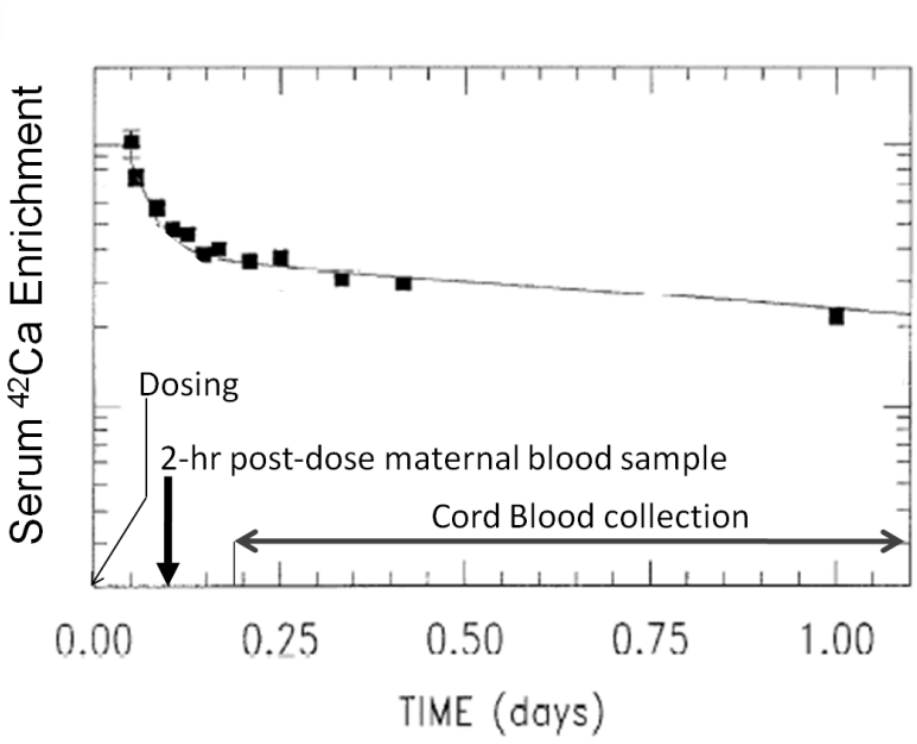

Figure 5.2 Timing of Two-Hour Post-Dosing Maternal and Cord Blood Sample Collection Superimposed on a Serum Enrichment Disappearance Curve
Shown above is a representative serum clearance curve of ^{42}Ca in a group of non-pregnant adolescent girls after intravenous administration of ^{42}Ca, as previously published by Wastney et al. (28). Superimposed on this curve are the time intervals in which the two-hour post-dose maternal blood collection, and cord blood collection were obtained in our cohort (n = 12).

After dosing, the serum enrichment of the intravenous tracer follows an exponential disappearance curve (**Figure 5.2**) (27-29). After administration of an oral isotope, the enrichment in serum increases as Ca is absorbed from the gut and tends to peak at approximately 2 h, and then exponentially disappears following a similar disappearance curve as noted for the intravenous tracer (28;30). For this reason, and to avoid sampling once active labor had begun, maternal blood was collected at 2 hours post-dosing. The intravenous tracer is, in essence, 100% absorbed and is administered to control for variability in the overall size and rates of loss from the miscible Ca pool. We followed a classic method used in studies that estimate fractional Ca absorption by administering an oral and intravenous isotope, and then generating a ratio of the oral to intravenous enrichment corrected for dose and natural abundance (25;31). We used the ratio of the enrichment of the oral isotope (^{44}Ca) to the enrichment of the intravenous isotope (^{42}Ca) in maternal circulation at two hours post-dosing, corrected for dose and natural abundance of these isotopes, to provide a relative measure of maternal Ca absorption (25), as follows:

$$\text{Relative maternal absorption} = \frac{(^{44}\text{Ca NA}) \times (^{44}\text{Ca}_{(M)}) \times (\text{mg } ^{42}\text{Ca given})}{(^{42}\text{Ca NA}) \times (^{42}\text{Ca}_{(M)}) \times (\text{mg } ^{44}\text{Ca given})} \quad \text{(Eq. 2)}$$

Modeling Determinants of Neonatal Enrichment and Placental Ca Transfer

We generated a quantitative estimate of maternal-to-fetal transfer of the ^{42}Ca and ^{44}Ca dose by calculating a ratio of the enrichment of isotope in the neonate at birth relative to the enrichment in the adolescent 2 h post-dosing. This calculation and the abbreviations used are shown below:

$$\text{Materal-fetal Ca transfer of } ^{42}\text{Ca} = \mathbf{^{42}Ca_{(C/M)}} = \frac{^{42}\text{Ca}_{(C)}}{^{42}\text{Ca}_{(M)}} \quad \text{(Eq. 3)}$$

$$\text{Materal-fetal Ca transfer of } {}^{44}\text{Ca} = \mathbf{{}^{44}Ca_{(C/M)}} = \frac{{}^{44}\text{Ca}_{(C)}}{{}^{44}\text{Ca}_{(M)}} \qquad \text{(Eq. 4)}$$

We then determined which study variables were associated with neonatal enrichment ($^{42}Ca_{(C)}$ and $^{44}Ca_{(C)}$, Eq. 1) and with the ratio of neonatal to maternal enrichment ($^{44}Ca_{(C/M)}$ and $^{44}Ca_{(C/M)}$, Eq. 3, 4).

Because the time elapsed from isotope administration to cord blood collection is a major determinant of cord blood enrichment, we controlled for the variation in this time-elapsed in two different ways, depending on whether isotopic measures were being used as outcome or predictor variables. When modeling determinants of neonatal enrichment, $^{42}Ca_{(C)}$ and $^{44}Ca_{(C)}$ were outcome variables. The best fit for a model of time (from dosing to cord blood collection) predicting $^{42}Ca_{(C)}$ and $^{44}Ca_{(C)}$ was determined to be log(time) verses enrichment, as shown below for $^{42}Ca_{(C)}$ in **Figure 5.3A**. Thus, to control for this time elapsed, potential predictor variables were individually entered into a multiple linear regression model as a covariate along with log (time from dosing to delivery), to determine if they were associated with enrichment in the neonate while controlling for time elapsed from dosing to delivery. Variables found to be significantly related to $^{42}Ca_{(C)}$ or $^{44}Ca_{(C)}$, while controlling for log(time to delivery), were considered predictors of neonatal Ca enrichment. Statistical determinants of maternal-fetal Ca transfer ($^{42}Ca_{(C/M)}$ and $^{44}Ca_{(C/M)}$; Eq. 3, 4) were also modeled, controlling for time in the exact same manner.

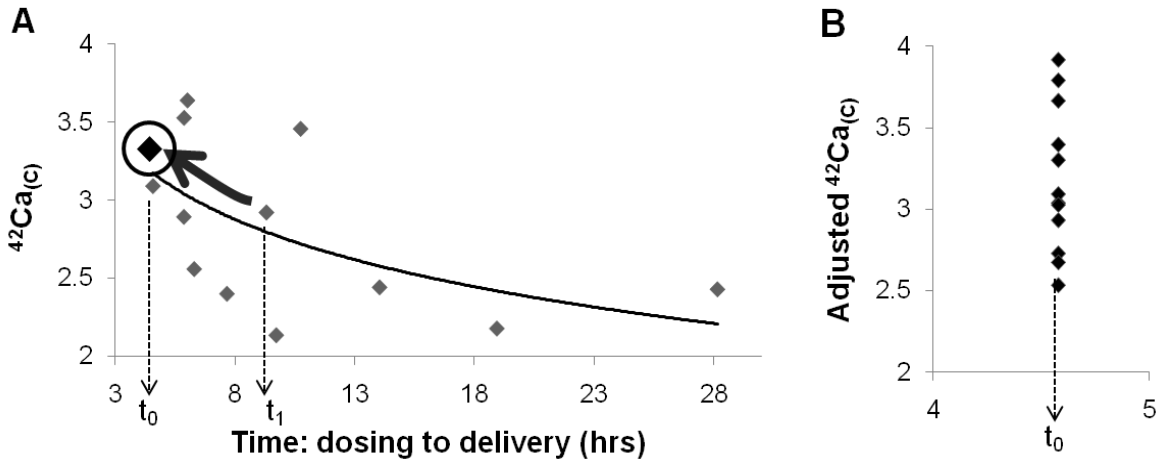

Figure 5.3 Generation of Time-Adjusted Neonatal Enrichment to Account for Variation in Time from Dosing to Time of Cord Blood Collection
A. Log**(**Time from dosing to delivery) is inversely related to ^{42}Ca enrichment in cord blood ($^{42}Ca_{(C)}$; $p = 0.069$, $R^2 = -0.294$). To adjust for time to delivery, all initial enrichment points at t_1 were extrapolated back to where they would fall on the log(time) curve at t_0: (the earliest cord collection; 4.58 hours), as shown with the circled dark point. B. The extrapolated ^{42}Ca enrichments in cord blood at t_0 ($^{42}Ca_{(Adj: C)}$) following adjustment for time to delivery are normally distributed.

Assessment of Relationships between Maternal-to-Fetal Ca Transfer and Neonatal Outcomes

When testing if maternal-fetal Ca transfer was associated with neonatal bone outcomes, $^{42}Ca_{(C/M)}$ and $^{44}Ca_{(C/M)}$ were used as predictor variables. In this case, entering log(time) as a covariate predictor of neonatal outcomes was inappropriate. Thus, we needed to adjust the measure of $^{42}Ca_{(C/M)}$ and $^{44}Ca_{(C/M)}$ to control for the variation in time elapsed from maternal dosing to cord blood collection in order to determine more accurately if maternal-fetal Ca transfer was associated with neonatal outcomes. We generated a measure of Ca transfer that accounted for time elapsed from dosing to delivery, and maternal enrichment. We then used this new variable as an independent variable to test for associations with neonatal outcomes.

To derive this adjusted measure of Ca transfer we calculated an "adjusted", $^{42}Ca_{(C)}$ ($\mathbf{^{42}Ca_{(Adj:\ C)}}$) as the enrichment of neonatal circulation at t_0 (the time of the earliest delivery), in order to control for differences in time from dosing to cord blood collection. The slope of the curve of log(time) vs. enrichment was used to extrapolate back to obtain $^{42}Ca_{(Adj:\ C)}$ at t_0, using $^{42}Ca_{(C)}$ observed at t_1 (the time elapsed from dosing to delivery) as initial points. A diagram of this generation of time-adjusted neonatal ^{42}Ca enrichment is shown above in **Figure 5.3B**. The equation derived from the log(time from dosing to delivery) vs. $^{42}Ca_{(C)}$ curve (**Figure 5.3A**) used to calculate this extrapolated $^{42}Ca_{(Adj:\ C)}$ enrichment was:

$$\mathbf{^{42}Ca_{(Adj:\ C)}} = m \times \log(t_0) + (^{42}Ca_{(C)} - (m \times \log(t_1)) \quad \text{(Eq. 5)}$$

t_0 = time from dosing to the fastest delivery = 4.58 hours

t_1 = time from dosing to delivery

m = slope of: $^{42}Ca_{(C)}$ vs. log (time from dosing to delivery) = -0.532219

The same approach was used to extrapolate back to obtain $^{44}Ca_{(C)}$ extrapolated to t_0 ($\mathbf{^{44}Ca_{(Adj:\ C)}}$), using $^{44}Ca_{(C)}$ and the time from dosing to delivery (t_1) as initial points:

$$\mathbf{^{44}Ca_{(Adj:\ C)}} = m \times \log(t_0) + (^{44}Ca_{(C)} - (m \times \log(t_1)) \quad \text{(Eq. 6)}$$

t_0 = time from dosing to the fastest delivery = 4.58 hours

t_1 = time from dosing to delivery

m = slope of: $^{44}Ca_{(C)}$ vs. log (time from dosing to delivery) = -0.615411

To derive a measure of time-adjusted maternal-fetal Ca transfer that also controlled for maternal enrichment, we simply generated a ratio of $^{42}Ca_{(Adj:\ C)}$ to maternal enrichment at 2 hours ($^{42}Ca_{(M)}$). The equation for this time-adjusted measure of Ca transfer was calculated as follows:

Time-adjusted maternal-fetal Ca transfer of ^{42}Ca = $\mathbf{^{42}Ca_{(Adj:\ C/M)}}$ = $\frac{^{42}Ca_{(Adj:\ C)}}{^{42}Ca_{(M)}}$ (Eq. 7)

Time-adjusted maternal-fetal Ca transfer of ^{44}Ca = $\mathbf{^{44}Ca_{(Adj:\ C/M)}}$ = $\frac{^{44}Ca_{(Adj:\ C)}}{^{44}Ca_{(M)}}$ (Eq. 8)

Simple linear regression was used to determine if $^{42}Ca_{(Adj:\ C/M)}$ or $^{44}Ca_{(Adj:\ C/M)}$ was related to fetal femur or humerus Z-scores or birth length, and multiple linear regression was used to test the relationship while controlling for other known predictors of fetal femur or humerus Z-scores previously identified in this population: maternal race, height, weight gain, Ca intake, vitamin D status, placental weight and placental VDR expression (Chapter 3, 4).

Statistical Analyses

Analyses were performed using SAS V. 9.2 and JMP 8.0 (SAS Institute Inc, Cary, NC). Results are reported as mean ± standard deviation (SD) unless otherwise stated. ANOVA was used to determine if categorical variables were related to maternal enrichment, neonatal enrichment and maternal-fetal Ca transfer. Simple linear regression was used to evaluate potential linear statistical predictors of estimated maternal Ca utilization, neonatal enrichment and maternal-fetal Ca transfer. Multiple linear regression was used to evaluate predictors of neonatal enrichment and $Ca_{(C/M)}$, always including log (time from dosing to delivery) as a covariate. Variables that were significant with log (time) as a covariate were considered predictors. If log (time) was not significant in the model, the p-value for the simple correlation was reported.

When considering maternal-fetal Ca transfer as a predictor of fetal outcomes, simple linear regression was used to determine if the time-adjusted measures of maternal-fetal Ca transfer ($^{42}Ca_{(Adj:C/M)}$ and $^{44}Ca_{(Adj: C/M)}$; Eq. 7, 8) were related to fetal outcomes. Multiple linear regression was also used to control for other predictors of fetal long bone length. In all modeling, variables that were not normally distributed were log transformed as necessary to assure normality of models' residuals. Normality was assessed using the Shapiro-Wilks test.

RESULTS

Subject Characteristics

Characteristics of the participants and their neonates are presented below in **Table 5.1**.

Table 5.1. Characteristics of Pregnant Adolescents (n = 12)

Characteristic	**Mean ± SD**
Maternal Age (years)	17.3 ± 0.7
African American Caucasian	75% 25%
Calcium Intake (mg/day)	694 ± 230
Pre-Pregnancy BMI (kg/m^2)	28.44 ± 6.38
Maternal Height (m)	1.58 ± 0.07
Weight Gain (lbs)	44.6 ± 23.3
Gestational Age Delivery (weeks)	39.9 ± 1.3
Infant Sex (male)	67%
Birth Weight (g)	3393 ± 426 g
Fetal Femur Length Z-Score	-0.314 ± 1.201
Fetal Humerus Length Z-Score	-0.457 ± 0.972

Maternal age at entry into the study ranged from 16.5 to 18.6 years. Reported Ca intake did not differ within subjects at different study visits, and thus the mean of reported Ca intakes was used (Chapter 1). This ranged from 270 to 1110 mg/day, with only 17% meeting the EAR (1100

mg/day), and none meeting the RDA (1300 mg/day) (32). The dietary Ca intake observed in this group was non-significantly lower ($p = 0.063$) than was observed in the larger cohort of pregnant adolescents from which these twelve teens were recruited (917 ± 416 mg/day, n = 163). In this smaller group, there was a wide range in both pre-pregnancy BMI (18.8 – 42.1 kg/m^2), and gestational weight gain (6.0 – 95.0 lbs). Both pre-pregnancy BMI and gestational weight gain were significantly higher than the means observed in the larger pregnant adolescent cohort (24.7 ± 5.5 kg/m^2, $p = 0.019$ and 37.1 ± 1.5 lbs, $p < 0.0001$, respectively). No other characteristics presented in **Table 5.1** significantly differed from characteristics of the larger cohort. Infant birth weight ranged from 2503 to 3915 g; all infants were healthy upon delivery and had no antenatal complications. Total serum Ca and calcitropic hormones in study participants and their neonates are presented below in **Table 5.2**.

Table 5.2.

Total Serum Ca and Calcitropic Hormone Concentrations in Maternal and Cord Blood[1]

	Mid-Gestation (28.4 ± 2.7 weeks)	**Delivery (39.9 ± 1.3 weeks)**	**Cord Blood**
25(OH)D (ng/mL)	17.6 ± 8.5[a]	18.4 ± 8.9[a]	17.5 ± 9.2[a]
PTH (pg/mL)	17.5 ± 6.4[a]	33.7 ± 14.3[b]	5.8 ± 0.5[c]
1,25(OH)$_2$D (pg/mL)	-	112.8 ± 37.2	-
Serum Ca (mg/dL)	9.0 ± 0.3[a]	9.0 ± 0.3[a]	10.5 ± 0.7[b]

[1] All values presented as mean ± SD.
[a, b, c] Different letter superscripts indicate that values significantly differed in paired analysis: $p < 0.008$

Approximately 75% and 50% of the adolescents were vitamin D insufficient (25(OH)D ≤ 20 ng/mL) at mid-gestation and delivery, respectively. Ten of the 12 adolescents were offered vitamin D supplements due to insufficient 25(OH)D concentrations observed at mid-gestation. Only eight adolescents received supplements, as two failed to return to the clinic after their mid-gestation blood work was complete. Only two adolescents self-reported consuming these vitamin D supplements on a daily basis over the remainder of gestation. Concentrations of 25(OH)D and the prevalence of 25(OH)D insufficiency at delivery did not differ between adolescents who were provided vitamin D supplements verses those who were not. Cord blood 25(OH)D did not statistically differ from maternal 25(OH)D at mid-gestation or delivery, and 73% of neonates were vitamin D insufficient. Adolescents who received vitamin D supplements were more likely to deliver neonates with 25(OH)D ≤ 20 ng/mL ($p = 0.004$). Serum PTH was elevated (PTH > 46 pg/mL) in two of the adolescents (17%) at delivery. PTH in cord blood was unrelated to maternal concentrations at either time point.

Dosage and Enrichment Obtained

The oral and intravenous doses administered are shown in **Table 5.3**. The oral dose represented ~0.052% of the miscible Ca pool size reported in pregnant women between 35 – 40 weeks; the intravenous dose represented ~0.021% of the miscible Ca pool size (4). The average time from dosing to the post-dose maternal blood draw was 2.11 ± 0.13 hours. Time elapsed from dosing to cord blood collection was 8.41 ± 6.96 hours (range: 4.58 – 28.17 hours). Average enrichments (Δ-% excess) observed in maternal and neonatal samples are presented below in **Table 5.3**, as are the ratio of fetal to maternal enrichments, the adjusted enrichments of the neonate at t_0, as well as the ratio of the time-adjusted neonatal to maternal enrichments.

The estimates of maternal-fetal Ca transfer did not significantly differ between the intravenous and oral isotope ($^{42}Ca_{(C/M)}$ vs. $^{44}Ca_{(C/M)}$; p = 0.17); nor did the time adjusted estimates of maternal-fetal Ca transfer ($^{42}Ca_{(Adj:C/M)}$ vs. $^{44}Ca_{(Adj: C/M)}$; p = 0.95).

Table 5.3.

Enrichment of Maternal Serum 2h Post-Dosing and of Cord Blood at Delivery[1]

Enrichment Value	**Abbreviation**	**Eq.**	**^{42}Ca**	**^{44}Ca**
Dose Delivered (mg)	-		1.062 ± 0.017	2.659 ± 0.050
Maternal serum Enrichment (%)	$Ca_{(M)}$	1	4.963 ± 0.987	2.608 ± 1.567
Neonatal Cord Enrichment (%)	$Ca_{(C)}$	1	2.807 ± 0.531	1.673 ± 0.832
Maternal-fetal Ca Transfer	$Ca_{(C/M)} = \frac{Ca_{(C)}}{Ca_{(M)}}$	3, 4	0.570 ± 0.056	0.881 ± 0.780
Neonatal Enrichment at t_0 (%)	$Ca_{(Adj:C)}$	5, 6	3.174 ± 0.446	2.097 ± 0.762
Time-Adjusted Maternal-fetal Ca Transfer	$Ca_{(Adj: C/M)} = \frac{Ca_{(Adj:C)}}{Ca_{(M)}}$	7, 8	0.650 ± 0.083	1.558 ±1.911

[1] All values presented as mean ± SD.

Predictors of Maternal Enrichment and Ca Absorption

Because the miscible Ca pool size is proportional to body size, enrichment of serum after dosing is inversely related to body size. As expected, the enrichment of ^{42}Ca in maternal

circulation ($^{42}Ca_{(M)}$) was significantly inversely related to pre-pregnancy weight ($p = 0.023$), weight at delivery ($p = 0.013$), BMI at delivery ($p = 0.038$), and estimated maternal blood volume ($p = 0.037$). A trend was present for a negative association between $^{42}Ca_{(M)}$ and maternal leptin concentrations at mid-gestation ($p = 0.104$) and at delivery ($p = 0.119$). Enrichment of ^{44}Ca in maternal circulation ($^{44}Ca_{(M)}$) was also significantly inversely related to maternal weight at delivery ($p = 0.033$) and leptin at mid-gestation ($p = 0.046$), and tended to be inversely associated with BMI at delivery ($p = 0.094$) and leptin at delivery ($p = 0.080$). Maternal Ca intake during pregnancy was significantly negatively associated with $^{44}Ca_{(M)}$ ($p = 0.029$).

Maternal Ca intake was also marginally negatively correlated with the relative measure of maternal Ca utilization (Eq. 2; $p = 0.066$, $R^2 = 0.30$). Maternal utilization of the oral tracer tended to be higher in the African American teens compared to the Caucasian participants ($p = 0.091$). Maternal Ca utilization was not related to any measure of maternal body size (pre-pregnancy weight and BMI, height, weight gain, weight or BMI at delivery, blood volume, etc.), or 25(OH)D, PTH, or $1,25(OH)_2D$ at any time point.

Predictors of Neonatal Enrichment & Maternal-Fetal Ca Transfer

The log(time from dosing to delivery) was used as a covariate when testing potential predictors of neonatal enrichment. **Table 5.4A** shows variables found to be significantly related to Ca enrichment in the neonate at delivery, controlling for log(time). As expected, the strongest predictor of $^{42}Ca_{(C)}$ was $^{42}Ca_{(M)}$. The strongest predictor of $^{44}Ca_{(C)}$ was maternal height. Maternal Ca intake was significantly inversely related to $^{44}Ca_{(C)}$ ($p = 0.040$), even though $^{44}Ca_{(C)}$ was not related to the estimate of maternal ^{44}Ca utilization ($p > 0.51$). All of the other predictors of $^{42}Ca_{(C)}$ and $^{44}Ca_{(C)}$, were reflective of maternal body size and were inversely related to

neonatal enrichment. No measures of 25(OH)D, PTH, or $1,25(OH)_2D$ in maternal or neonatal circulation were related to neonatal enrichment. Other variables that were unrelated to measures of neonatal enrichment included: race, gestational weight gain, season of delivery, vitamin D intake, infant size, and serum Ca.

Statistical predictors of maternal-fetal Ca transfer ($Ca_{(C/M)}$; Eq. 3, 4), controlling for log(time to delivery), are shown in **Table 5.4B**. Maternal vitamin D intake, maternal 25(OH)D sufficiency at mid-gestation, and placental VDR expression were significantly positively associated with $^{42}Ca_{(C/M)}$. Variables not shown in **Table 5.4B** that were unrelated to either $^{42}Ca_{(C/M)}$ or $^{44}Ca_{(C/M)}$ include: PTH, $1,25(OH)_2D$, maternal weight, height, weight gain, and BMI, season of delivery, infant size, and serum Ca.

Table 5.4

Predictors of Neonatal Enrichment at Birth ($^{42}Ca_{(C)}$ and $^{44}Ca_{(C)}$) and Maternal-Fetal Ca Transfer ($^{42}Ca_{(C/M)}$ and $^{44}Ca_{(C/M)}$), Controlling for Time-to-Delivery

A.	**$^{42}Ca_{(C)}$**		**$^{44}Ca_{(C)}$**	
Predictor	**p**	**r**	**p**	**r**
Ca Intake (mg/d)	0.8904[2]	NA	0.0399[1]	-0.45
Maternal Enrichment	<0.0001[1]	0.88	0.0755[1]	0.43
Maternal Leptin at Mid-Gestation	0.0165[1]	-0.67	0.0981[2]	-0.50
Maternal Leptin at Delivery	0.0276[1]	-0.72	0.1710[2]	-0.42
Pre-Pregnancy BMI	0.0555[1]	-0.66	0.3922[2]	NA
Pre-Pregnant Weight	0.0068[1]	-0.67	0.1206[2]	-0.47
Maternal Height	0.0371[1]	-0.41	0.0044[1]	-0.62
Maternal Weight at Delivery	0.0027[1]	-0.52	0.1316[2]	-0.46
Maternal BMI at Delivery	0.0172[1]	-0.67	0.3887[2]	NA
Estimated Maternal BV at Delivery	0.0045[1]	-0.59	0.0651[1]	-0.46
B.	**$^{42}Ca_{(C/M)}$**		**$^{44}Ca_{(C/M)}$**	
Predictor	p	r	p	r
Vitamin D intake	0.0527[2]	0.57	0.8309[2]	NA
Sufficient 25(OH)D at Mid-gestation	0.0490[2]	Higher $^{42}Ca_{(C/M)}$	0.3067[2]	NA
Maternal Ca Utilization (Eq. 2)	0.5897[2]	NA	0.0481[2]	-0.60
Placental VDR Expression	0.0042[2]	0.64	0.6038[2]	NA

NA = Not applicable: no r value given for p > 0.30; BV = Blood volume
[1] = p-value given is for parameter estimate when controlling for log(time to delivery)
[2] = controlling for log(time to delivery) does not change correlation

A multiple regression model of $^{42}Ca_{(C/M)}$, was generated that included the variables identified above as significant covariates, as well as log(time to delivery), placental weight and an interaction between placental weight and VDR expression as initial covariates. After non-significant predictors were removed from the model, log(time), placental VDR expression, and the interaction between placental weight and VDR expression remained significant covariates in the model of $^{42}Ca_{(C/M)}$, and together explained 80.0% of the observed variation in this measure of maternal-fetal transfer of ^{42}Ca. A similar approach was taken to model determinants of

maternal-fetal transfer of ^{44}Ca ($^{44}Ca_{(C/M)}$); estimated maternal Ca utilization (Eq. 2) alone remained a significant predictor.

Associations with Neonatal Bone Outcomes

To determine if maternal-fetal Ca transfer was related to fetal and neonatal skeletal outcomes, the time-adjusted measures of maternal-fetal Ca transfer ($^{42}Ca_{(Adj: C/M)}$ and $^{44}Ca_{(Adj: C/M)}$; Eq. 7, 8) were tested for association with fetal femur Z-score, fetal humerus Z-score, and birth length. There were no significant relationships between either $^{42}Ca_{(Adj: C/M)}$ or $^{44}Ca_{(Adj: C/M)}$ and any of these skeletal outcomes. However, when models of fetal femur and humerus Z-scores were generated that included maternal race, height, weight gain, Ca intake, vitamin D status, placental weight and placental VDR expression as covariates, significant relationships between $^{42}Ca_{(Adj: C/M)}$ and $^{44}Ca_{(Adj: C/M)}$ and fetal skeletal outcomes became evident. The best model of fetal femur Z-score in this cohort included the variables of maternal weight gain, maternal 25(OH)D at delivery, placental weight, placental VDR expression, and $^{42}Ca_{(Adj: C/M)}$. Together these variables explained 82.0% of the variation in fetal femur Z-scores ($p = 0.013$). The addition of $^{42}Ca_{(Adj: C/M)}$ to this model of the model for fetal femur Z-score increased the R^2 from 0.58 to 0.82. The best model of fetal humerus Z-score in this cohort included the variables of maternal race, height, weight gain, Ca intake and 25(OH)D at delivery, placental weight, placental VDR expression, the interaction between placental weight and VDR expression, and $^{44}Ca_{(Adj: C/M)}$. Together these variables explained 99.9% of the variation in fetal humerus Z-scores ($p = 0.018$). The addition of $^{44}Ca_{(Adj: C/M)}$ to this model of fetal humerus Z-score increased the R^2 by from 0.46 to 0.99.

DISCUSSION

In this research we utilized a novel approach to obtain in-vivo measures of maternal-to-fetal Ca transport in human pregnancy. The data obtained inform our understanding of Ca dynamics during pregnancy, and suggest that maternal vitamin D status and placental expression of the vitamin D receptor (VDR) may play a role in regulation of Ca transport to the fetus. Additionally, the measures of maternal-fetal Ca transfer that we derived were significant in models of fetal femur and humerus length Z-scores, suggesting that increases in Ca transport to the fetus are incorporated into the developing fetal skeleton, and reflected in long bone length.

We utilized a novel approach, administering dual-stable Ca isotopes to pregnant adolescents early in labor and assessing enrichment in maternal circulation post-dosing, and in cord blood at birth. The main predictor of neonatal ^{42}Ca enrichment was maternal ^{42}Ca enrichment. When characterizing enrichment of maternal serum post-dosing, the main statistical predictors of maternal enrichment were measures of maternal body size and leptin. These measures were also negatively associated with enrichment in cord blood. Serum leptin is reflective of adiposity and may be as a proxy measure of fatness. However, leptin also has physiological properties, plays a role in maintaining energy balance during pregnancy (33), and has been shown to increase more-so in pregnant adolescents that are still growing, than in adult pregnancies (34). It is unknown whether the negative association we detected between maternal serum leptin and Ca enrichment is simply a reflection of body size, or indicative of an alternate physiological role of leptin during pregnancy.

We derived estimates of maternal-fetal Ca transfer by generating a ratio of neonatal to maternal enrichment ($^{42}Ca_{(C/M)}$ and $^{44}Ca_{(C/M)}$) and these ratio estimates of maternal-fetal Ca transport of the oral and intravenous doses did not significantly differ. This implies that there is

not preferential maternal-fetal transfer of Ca derived from a meal over Ca in the miscible Ca pool originating from other inputs, such as maternal bone turnover. We report here that maternal vitamin D sufficiency at mid-gestation was positively associated with maternal-fetal ^{42}Ca transport ($^{42}Ca_{(C/M)}$). Our findings imply that maternal 25(OH)D sufficiency is associated with increased maternal-fetal transport of Ca to the fetus, which provides biological plausibility to the body of literature that links maternal 25(OH)D sufficiency with indices of fetal and infant bone (35), including fetal femoral development (15), and knee-to-heel length at birth (16). We also report here that placental VDR expression is significant in models of maternal-fetal ^{42}Ca transport ($^{42}Ca_{(C/M)}$). This positive relationship is also biologically plausible, as the expression of many Ca transporters in the placenta are regulated by VDR-mediated $1,25(OH)_2D$ activity (36). Increased VDR expression may increase expression of these transporters impacting net Ca transfer.

In this small sample size, we observed that the time-adjusted estimates of maternal-fetal Ca transfer were associated with significantly more positive fetal femur and humerus length Z-scores in multivariate models. As Ca is the main mineral component of bone, is necessary for skeletal mineralization, and also plays a role in regulation of endochondral bone growth (37-39), it follows that increased transport of Ca to the fetus may result in increased long bone length.

In the larger cohort of pregnant adolescents (from whom these participants were recruited) we have shown that placental VDR expression is a significant positive covariate in models of fetal femur Z-score (Chapter 4). In this sub-study we show that placental VDR expression may regulate maternal-fetal Ca transport, which impacts fetal femur and humerus length. These finding provide a physiological explanation of the association detected between VDR expression and fetal femur Z-score in the larger cohort (Chapter 4). It is noteworthy that

adding our measure of maternal-fetal Ca transfer to models of fetal femur and humerus Z-scores resulted in a large increase in the R^2 of the models. Our sample size is quite limited and thus the modeling in this study is not nearly as powerful as the modeling of fetal femur Z-scores in the larger cohort (Chapters 3, 4). Thus, the model characteristics presented here must be interpreted with caution. However, it is still particularly noteworthy that the addition of this dynamic measure of actual in-vivo Ca transport ($^{42}Ca_{(Adj:\ C/M)}$ and $^{44}Ca_{(Adj:\ C/M)}$) to the other covariates (which are all static measures of maternal or placental characteristics) dramatically improved the predictive capacity of these models. As fetal Ca demand and placental Ca transfer increases late in gestation (3), we may have improved our ability to detect differences in Ca transport by assessing this measure at term. Furthermore, the potential competition for nutrients between maternal and fetal needs, combined with the low Ca intake and 25(OH)D status observed in this group of 12, may have also increased our ability to detect statistical relationships indicative of factors regulating placental Ca transport under suboptimal maternal availability.

The dual isotope approach used in this study allowed us to generate a relative measure of maternal utilization of the oral dose. Because we were only able to assess maternal enrichment at a single time-point, we were unable to calculate actual fractional Ca absorption. However comparing the adjusted ratio of ^{44}Ca to ^{42}Ca in maternal circulation (Eq. 2) provides a relative estimate of maternal dietary Ca utilization between participants. In this group, this relative measure of maternal Ca utilization tended to be higher in African American vs. Caucasian adolescents ($p = 0.091$). Non-pregnant African American girls (age 5 – 16 years) exhibit higher rates of fraction and total Ca absorption compared to Caucasian counterparts (40). The same racial dichotomy may be observed in this study. Maternal Ca utilization in this group was also inversely related to habitual Ca intake. This relationships is not commonly detected in pregnant

populations (41-43), as fractional Ca absorption universally increases during pregnancy (4) even if Ca intake increases (41). However, the inverse relationship between Ca intake and fractional Ca absorption is well documented among non-pregnant populations and non-pregnant adolescents (40;44). The wide range in Ca intakes (270 to 1110 mg/day) in this group may have increased our ability to detect this association. However, as our sample size was limited, the detection of such novel associations must be interpreted with caution. Other innate factors in our study design that were beyond our control may have added error to our relative measure of maternal Ca utilization. For instance, even though adolescents were not provided food upon admittance to the hospital, they arrived in various states of fasting. Thus the contents of the gut, which impacts Ca absorption, cannot be controlled for. Furthermore, this study was undertaken during labor, and it is not known to what degree intestinal Ca absorption is impacted by the physiological and hormonal state of parturition.

This study is novel in its approach to obtain in-vivo measures of placental Ca partitioning in human pregnancy. To our knowledge, this has never been attempted. However, there were several limitations to this methodology that arose from the variable timing and unpredictable nature of delivery. We controlled for time-to-delivery statistically, but were unable to control for the stage of labor at which the post-dosing maternal blood collection was obtained. Undertaking this approach in women undergoing planned cesarean sections may allow for more accurate control over these variables. However, planned surgical removal of the fetus introduces different stressors on the system that may impact results. Neither approach is without limitations. A larger sample size would also have increased our power to detect relationships and more accurately model determinants of maternal-fetal Ca transfer.

Despite these limitations, this study informs our understanding of placental Ca transport in humans. We have shown that maternal 25(OH)D status and placental expression of VDR are associated with measures of maternal-fetal Ca transport. This data suggests that increases in transport of Ca are incorporated into the fetal skeleton and reflected in fetal long bone length.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Summary

This doctoral research describes and discusses novel findings regarding the impact of Ca intake and vitamin D status on calcitropic hormones during pregnancy, on indices of fetal and infant skeletal health, and on vitamin D receptor (VDR) expression in the placenta - in the context of adolescent pregnancy. This work also reports findings generated by a novel approach to quantify placental Ca transfer in-vivo, and discusses the impact of maternal Ca intake and vitamin D status on this process. There were four specific aims of this dissertation that were individually detailed and discussed in chapters two through five. Below is a brief summary of the conclusions from each specific aim, followed by the implications of this research as a whole, and suggestions for future research.

Pregnancy induces a variety of maternal physiological adaptations that occur to accommodate the demands of the developing fetus. In order to increase Ca availability to the fetus and accommodate fetal skeletal growth, intestinal Ca absorption and metabolic bone activity increase significantly in the pregnant woman. These physiological adaptations are accompanied by changes in the calcitropic hormones: PTH and $1,25(OH)_2D$.

The first specific aim was to investigate how maternal Ca intake and 25(OH)D status were associated with temporal changes in calcitropic hormone concentrations across pregnancy in a cohort of pregnant adolescents. This age group is at risk for both inadequate Ca intake and vitamin D insufficiency and may have an elevated Ca demand due to the combined Ca required for both continued maternal growth and fetal skeletal mineralization. We detected a high prevalence of vitamin D insufficiency in these adolescents. We also reported that PTH increased across gestation, and was elevated ($\geq$ 46 pg/mL) in 40% of teens by delivery. As most studies of PTH during pregnancy report that circulating concentrations of this hormone do not change

and/or remain low, this finding is novel. Parathyroid hormone may be increased as a compensatory response to ensure adequate Ca supply to the fetus under conditions of a strained maternal Ca economy. Data from markers of bone turnover in this group (presented in Appendix 11) support this observation; adolescents who exhibited elevated PTH at delivery had significantly higher circulating concentrations of a marker of bone resorption when compared to adolescents with PTH concentrations within normal ranges. The inverse relationship detected between 25(OH)D and PTH at mid-gestation and delivery suggests that low vitamin D status may exacerbate a constrained ability to maintain Ca homeostasis when demands are elevated, and also indicates that limited 25(OH)D may be impacting the elevated concentrations of PTH observed. Serum 25(OH)D was also inversely related to $1,25(OH)_2D$ in a time-delayed manner, while PTH and Ca intake were not related to $1,25(OH)_2D$. Together these findings suggest that this cohort exhibits an increased Ca demand during pregnancy, and that maternal concentration of the vitamin D prohormone, 25(OH)D, was playing a significant role in these hormonal alterations. The observed changes were not associated with maternal age across the 13-18 year age range in this adolescent study population. Maternal Ca intake seemed to be less involved in regulation of these hormones at the relatively high Ca intakes observed (~ 1000 mg/day). To our knowledge this is among the largest published study to date to report on Ca intake and $1,25(OH)_2D$ concentrations across pregnancy and among neonates at birth.

Our second aim was to determine if maternal Ca intake and vitamin D status were associated with measures of fetal and infant skeletal health, and to determine if there was any interaction between these nutrients and fetal bone length. Both maternal Ca intake and vitamin D status were associated with fetal femur and humerus length Z-scores and with neonatal length at birth. Significant improvements in both fetal long bone Z-scores and birth length became

evident at a dietary Ca intake of 1050 mg/day. Adolescents with sufficient 25(OH)D (> 20 ng/mL) exhibited more positive fetal femur and humerus Z-scores. There was a significant interaction in the effects of Ca intake and 25(OH)D status that implied a capacity of one nutrient to compensate for limited intake/status of the other. These findings have clinical relevance in that they suggest that achieving adequacy in only one nutrient may have a significant impact on fetal skeletal growth that is maintained at delivery. This data underscores the importance of encouraging pregnant adolescents to meet dietary Ca intake recommendations and/or to achieve sufficient 25(OH)D status in order to ensure optimal fetal skeletal development.

Our third specific aim was to investigate how maternal Ca intake and vitamin D status were related to VDR expression in the placenta, and if variation in VDR expression was related to fetal femur Z-scores. Circulating concentrations of the hormonal form of vitamin D in the neonate ($1,25(OH)_2D$) were most strongly linearly associated with placental VDR expression. Neonatal 25(OH)D status was significantly inversely related to placental VDR, but the analogous relationship with maternal 25(OH)D only approached significance. Dietary Ca intake was not associated with placental VDR expression. These findings may suggest that placental VDR is increased as a compensatory response to limited neonatal 25(OH)D status, and is stimulated by circulating neonatal concentrations of $1,25(OH)_2D$. Maternal 25(OH)D was not significantly associated with neonatal $1,25(OH)_2D$, further strengthening the independent effects of fetal signals on placental VDR expression. Functional consequences of placental VDR expression were also evident as indicated by the finding that placental VDR expression was a significant predictor of fetal femur length Z-scores. Increased placental VDR expression may facilitate the placenta's capacity for vitamin D – mediated regulation of gene expression, which may impact rates of Ca transport to the fetus.

Our final specific aim was to obtain in-vivo measures of placental Ca transport and determine if maternal Ca intake, vitamin D status, or placental VDR expression was associated with Ca enrichment of maternally administered stable Ca isotopes in the neonate at birth. Placental VDR expression was found to be a statistically significant predictor of maternal-to-fetal transfer of intravenous isotope (^{42}Ca) to the fetus, but was not associated with maternal-to-fetal transfer of the maternally ingested isotope (^{44}Ca). The lack of a relationship with maternal-to-fetal transfer of the oral isotope is likely due to the additional variability in this measure that results from individual variation in intestinal Ca absorption– variability to which the intravenous dose is not subject. Maternal 25(OH)D status at mid-gestation (but not at delivery) was also associated with higher placental transfer of intravenous tracer to the neonate. These relationships suggest that placental VDR expression facilitates Ca transfer to the fetus *in-utero*, which corroborates the association previously detected between placental VDR expression and fetal femur length (Chapter 4). Finally, the in-vivo measures of maternal-to-fetal Ca transfer remained significant when entered into models of fetal femur and humerus length Z-scores linking our dynamic measures of placental Ca flux with static measures of fetal long-bone length in vivo.

The relationships detailed in this research are summarized below in **Figure 6.1**.

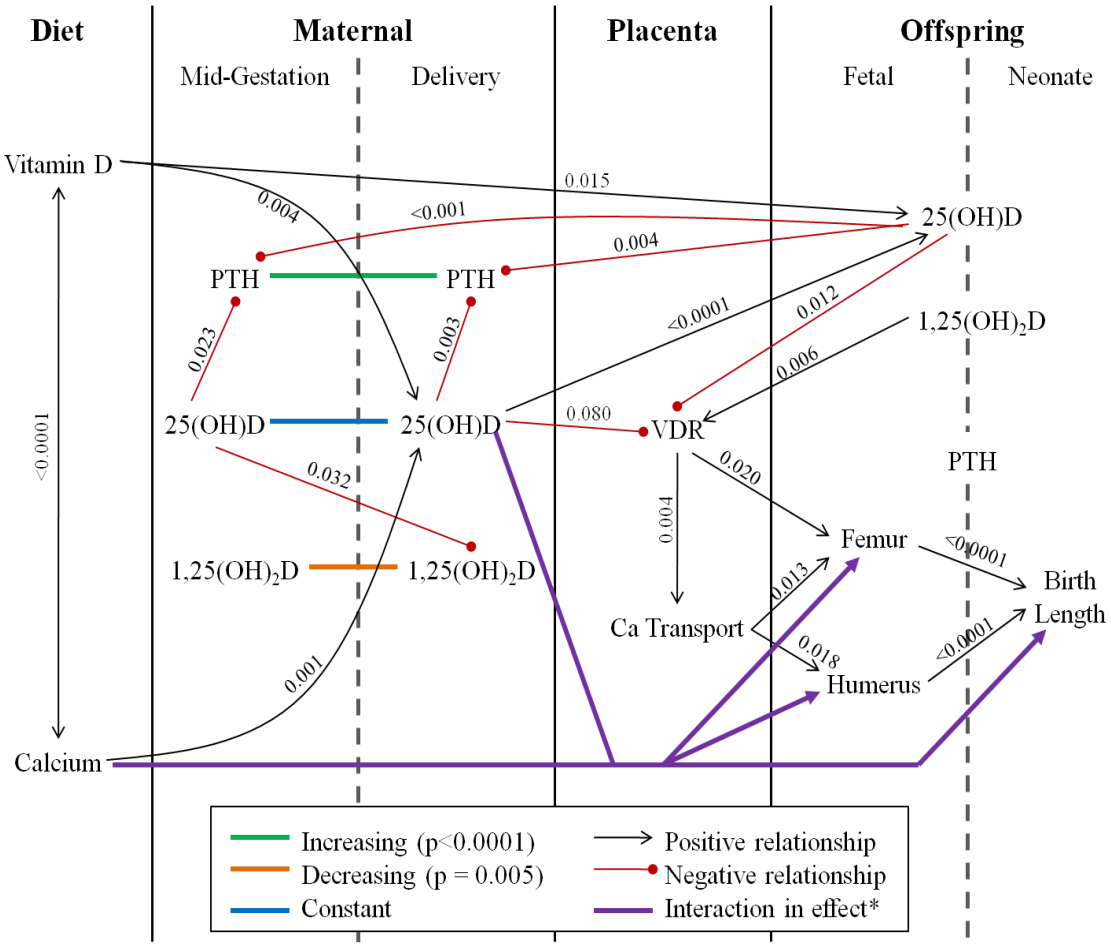

Figure 6.1 Summary of Relationships Documented In Doctoral Research
Summary of findings from chapters 2 – 5, indicating the significant relationships observed between maternal vitamin D status and maternal Ca intake on calcitropic hormones in the pregnant adolescent and neonate, placental expression of VDR, and fetal and neonatal skeletal outcomes. Corresponding p-values for each relationship are indicated. *The nature of the interaction indicated by the purple arrows, suggests that Ca intake is a significant predictor of fetal femur and humerus length and birth length only when maternal 25(OH)D ≤ 20 ng/mL. Similarly, maternal 25(OH)D sufficiency is a significant predictor of fetal femur and humerus length only when maternal Ca intake < 1050 mg/day.

When the data as a whole is examined in **Figure 6.1**, the number of arrows originating from maternal Ca intake alone vs. maternal 25(OH)D or Ca/vitamin D interactions make it clear that maternal vitamin D status is significantly associated with the calcitropic response to

pregnancy in these adolescents, more so than Ca intake. Furthermore, both maternal vitamin D status (25(OH)D) and both neonatal 25(OH)D and $1,25(OH)_2D$ impact placental VDR expression, which is associated with dynamic measures of maternal-to-fetal Ca transfer and gestational-age adjusted measures of fetal femur length *in-utero*. The modulation of placental VDR may be one mechanism by which maternal vitamin D status impacts fetal long bone Z-scores and neonatal birth length. Finally, the observed interaction between maternal Ca intake and vitamin D status on fetal and neonatal bone outcomes implies that the impact of vitamin D on fetal/neonatal outcomes is most significant when maternal dietary Ca intake is suboptimal and vice versa. This interaction is clinically relevant as it implies that significant improvements in fetal and neonatal bone outcomes can be obtained if the cohort of Ca/D insufficient pregnant adolescents can achieve either adequate Ca intake ($\geq$ 1050 mg/day) or sufficient 25(OH)D status.

Placental transport of nutrients and subsequent fetal development is a highly complex process that is regulated by both maternal and neonatal factors. Under conditions of suboptimal maternal Ca intake or inadequate vitamin D status, compensatory mechanisms, such as were observed in this study, may become active to ensure adequate nutrient supply to the fetus. The large range in Ca intakes observed, the prevalence of inadequate 25(OH)D detected in this population, and the skeletal immaturity of this age group likely improved our ability to detect many of the relationships noted in **Figure 6.1**. An additional strength of the study is the prospective longitudinal design, which provided us with an opportunity to address temporal trends in relevant calcitropic hormones. The assessment of maternal nutrient intake, biomarkers of nutritional status in the mother and neonate, and assessment of fetal and neonatal bone outcomes adds to the overall impact of these data.

In this research, the effect sizes observed in relation to fetal and neonatal bone outcomes (eg: maternal Ca intake, 25(OH)D sufficiency, and placental VDR expression, etc.) were relatively small, explaining on average less than 10% of the variation in outcomes noted. However, as fetal skeletal development is known to be a complex process and is regulated by both environmental and genetic factors, a model with a large R^2 was not expected. It is estimated that fully ¾ of bone mass is genetically determined and not modifiable and that individual variation in Ca nutrition early in life only explains 5 -10% of peak adult bone mass (1). This fits with the effect sizes described here. However, it is also known that bone mass "tracks" throughout life (1), and as such a 5% difference in bone mass if maintained, may have a dramatic impact on subsequent skeletal health. Existing data have found that even a 5 – 10% difference in peak adult bone mass translates into a 25 – 50% difference in hip fracture rate late in life (1). Thus, to identify easily modifiable factors (such as Ca intake and vitamin D status) that are significant predictors of fetal bone length at any effect size, is of clinical significance to this population.

The last month has witnessed a dramatic increase in the data available on calcitropic hormone and vitamin D status across gestation, and our data contribute to this increase in knowledge. A very large cross-sectional study (n = 1016) was published in July, 2011 by Haddow et al. addressing the relationship between 25(OH)D and PTH early in pregnancy (11 – 13 weeks) (2). The large sample size and racial diversity (42% African American) of this cohort has addressed a well-known gap in existing literature (2). Furthermore, just two weeks ago, Hollis et al. published the results of an NIH-funded, randomized vitamin D supplementation trial during pregnancy (n = 530) (3). Until this study was published, ours would have been the largest

report on 1,25$(OH)_2$D during pregnancy and in the neonate. It is still the largest to address these issues in pregnant adolescents.

Haddow et al., detected similar findings and a similar racial dichotomy regarding the negative relationship between 25(OH)D and PTH as we detected in our pregnant adolescents (2). However they did not undertake analyses grouping both races together as a function of 25(OH)D status (as we did), to examine if it was the racial or vitamin D status that was driving the relationships observed. In addition, their study was cross-sectional early in gestation at a time that fetal skeletal demands are low (11 -13 weeks of gestation), and therefore provides no data on PTH concentrations late in pregnancy, when fetal Ca demands are highest (2). Hollis et al. established the safety of large doses of vitamin D and found these to increase 25(OH)D status. These data also described relationships between 25(OH)D and both PTH and 1,25$(OH)_2$D across pregnancy (3). A large randomized clinical trial of vitamin D supplementation during pregnancy has been widely called for in the literature, but the Hollis et al study suffered fundamental flaws in the randomization process (due to IRB regulation) that make interpretation of the intention-to-treat analyses difficult. Furthermore, the relationships reported between 25(OH)D and the calcitropic hormones were observational in nature, based on an analyses of all supplementation groups combined. Thus, despite the study being a randomized controlled trial, the inferences drawn are not of stronger evidence than our own. Yet, despite these caveats, this study addresses a significant gap and adds to data on maternal calcitropic hormone physiology in adult women. Our study population was skeletally immature and vitamin D insufficient throughout pregnancy, and thus more likely to exhibit hormonal changes and relationships that are indicative of compensatory responses to meet fetal demand; such relationships may not be present or detectable in adult women. Studies that focus only on early gestation may miss these changes,

and studies that supplement with vitamin D result in improvements in 25(OH)D status that may independently impact homeostasis. Furthermore, our data differ from Hollis and Haddow as we took a holistic approach to address all three physiological participants of pregnancy: addressing status and outcomes in the mother, the fetus, and at the level of the placenta. As the placenta mediates nutrient transfer between the mother and fetus, our consideration of placental role in the maternal/fetal relationships observed increases the interpretation of our findings. We also addressed the impact of vitamin D status on functional skeletal outcome in the fetus and neonate, beyond neonatal status only. Finally, our studies increase knowledge regarding interactions between maternal Ca intake and vitamin D status during pregnancy – a point which both of these studies neglected to address.

Our assessment of fetal femur/humerus length and neonatal birth length were important study outcomes as it has become increasingly appreciated that nutritional status and growth *in-utero* impact the offspring's subsequent risk of chronic disease in adulthood. The links between maternal nutritional status and the fetal and neonatal skeletal measures described in this research contribute to the fetal programming literature, and to our understanding of fetal responses to maternal nutrient supply. Finally, these data are of clinical relevance, as the Ca intakes and vitamin D status observed in this cohort are representative of the consumption and status patterns of many pregnant women in the United States, and thus these findings have broader relevance to adult pregnant women with inadequate Ca intake and/or vitamin D status and highlight the need for further research in this area.

Limitations and Considerations

This research makes a legitimate contribution to our understanding of calcitropic hormone response and fetal programming in adolescent pregnancy. However, our study had several limitations that may be addressed in future research. As discussed in Chapter 2, there are limitations to assessing PTH status from a single blood sample that was not taken in the fasted state at a standardized time. There are also limitations to assessing habitual dietary intake using a 24 hour recall and food frequency questionnaire. Additional measures of maternal and neonatal BMC at birth would have strengthened study outcomes but proved too difficult to obtain logistically in this age group and clinical setting. It would have been preferable to have recruited only non-smoking teens but recruitment was expanded to maximize study numbers. The timing of our study measures was highly variable given compliance issues in this age group. In addition, concurrent adult data would have allowed us to more fully distinguish adolescent effects from those due to Ca and vitamin D insufficiency, but study funding limitations precluded these measures. Many of our variables were self-reported and may not be accurate in this group and there are no good biomarkers for Ca intake.

While there are many limitations and study design components that could have been improved upon, working with a pregnant adolescent population is extremely challenging, as they are a vulnerable research population, have limited independence, limited access to transportation and/or are not old enough to drive, and this age-group is often unreliable in terms of follow-up and self-reported data. The lack of post-partum follow up data on bone outcomes is a limitation to this research, and is called for in this population. The frequent change in permanent residence, and contact information made follow up in this particular cohort impossible. However, these data would inform our understanding of the sustained effects of maternal Ca intake and vitamin D status during adolescent pregnancy on subsequent maternal and offspring outcomes.

Future Directions

These data have provided novel insight into the impact of maternal vitamin D and Ca status during pregnancy on the calcitropic hormone response to pregnancy, placental expression of VDR, transfer of Ca to the fetus, and fetal bone growth. These findings have also generated new questions. Additional research on the impact of elevated PTH and low 25(OH)D on maternal bone mass across pregnancy are needed in this pediatric population. Given the significant interactions observed between dietary Ca and vitamin D status, additional studies assessing dietary interventions with controlled intakes of both nutrients should be explored. Studies are also warranted to determine if the fetal bone deficits observed *in-utero* and the birth length deficits observed at birth are maintained or if these deficits are influenced by subsequent diet or adaptations that occur in early growth. Studies assessing net skeletal accretion in the neonate at birth are also called for, as these assessments were not possible in this cohort.

This research has also identified novel relationships between neonatal vitamin D status and placental expression of VDR. Mechanistic studies are needed to examine the possible additional effects of increased VDR expression on other VDRE-containing genes in the placenta. Similarly, additional work is needed to fully explore $1,25(OH)_2D$ / VDR action in the placenta, given the known and varied endocrine, paracrine, and intracrine effects of this hormone in various tissues. Animal models may be particularly useful in investigating how the placental responds to fetal endocrine signals. Fetal calcitriol production can be up-regulated by altering the fetal genome and placental gene expression and Ca transport can be monitored in response to targeted manipulations of the system. Additional studies documenting the effect of maternal diet on the outcomes studied would also improve our understanding of the placental role in nutrient

partitioning and the mechanisms in place to regulate partitioning between the adolescent and fetus.

Finally, these results also call for more qualitative studies to understand the roots of the poor supplement adherence, unhealthy dietary patterns and extremes of gestational weight gain observed in these adolescents. Results from such work are necessary in order to design age-appropriate interventions that target at-risk teens early in pregnancy and provide adequate nutrition and education to ensure achievement of optimal nutrient status by term. Such research can improve maternal and birth outcomes in this vulnerable age group and ensure adequate neonatal endowment of key nutrients, which will result in immediate and possible long-term health gains.

REFERENCES

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APPENDIX 1

MATERNAL DEMOGRAPHIC DATA COLLECTION TELEFORM

4731378542

Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

Demographics

MRN: ☐☐☐ - ☐☐ - ☐☐ DOB: ☐☐ / ☐☐ / 19☐☐ mm/dd/yyyy

Gestational Age: ☐☐ weeks* as of ☐☐ / ☐☐ / 20☐☐ mm/dd/yyyy*

When was the first day of your last period? ☐☐ / ☐☐ / 20☐☐ mm/dd/yyyy*

When is your baby due? ☐☐ / ☐☐ / 20☐☐ mm/dd/yyyy*

During what month / week of this pregnancy did you first seek prenatal care? ☐☐ Month OR ☐☐ Week

What was your average weight before pregnancy? ☐☐☐ lbs* OR ☐☐☐ kgs*

How tall are you without shoes on? ☐ ft ☐☐ inches OR ☐☐☐ centimeters

How old were you when you had your first period? ☐☐ years old

Is this your first pregnancy?

- ○ Yes
- ○ Don't know / Not sure
- ○ NO... How many past pregnancies, including this one?** ☐☐
  - How many children have you given birth to?** ☐☐
  - How many abortions have you had?** ☐☐
  - How many miscarriages have you had?** ☐☐

Do you intend to breastfeed your child? ○ Yes ○ No ○ Don't know yet/ Not sure

Were you using birth control? ○ No ○ Yes, using this type: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Have you had any of the following problems currently or in the past?

○ Major Injuries ○ Auto Accidents ○ Broken bones ○ Bone disease ○ Joint Disease

Have you ever had a sexually transmitted disease (STD)?

- ○ No
- ○ Don't know / Not sure
- ○ **Yes, I have . . .** (bubble all that apply)
  - ○ Chlamydia ○ Bacterial vaginosis ○ Genital herpes ○ Syphilis
  - ○ Gonorrhea ○ Genital warts ○ HIV / AIDS ○ Trichomonas
  - ○ Other ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

*If unknown, code "?" (i.e. 11/??/2006) **If not applicable, leave empty.

2370378549

Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

Have any of your family members had any of the following conditions? (grandparents, parents, brothers or children)

			Relationship
Diabetes	○ Yes	○ No	
Heart Disease	○ Yes	○ No	
High Blood Pressure	○ Yes	○ No	
Osteoporosis (brittle bones)	○ Yes	○ No	
Other bone / joint disease	○ Yes	○ No	

Medication		Frequency	Date (mm/dd/yy)
	Dose / Unit		Start / Stop
	Dose / Unit		Start / Stop
	Dose / Unit		Start / Stop
	Dose / Unit		Start / Stop

○ Bubble here if there are more medications not listed.

Did you ever drink or currently drink alcohol?

○ Never

○ Currently
○ I Used To

drink, ☐☐.☐ Cups

○ Every day
○ Occasionally
○ 2 - 3 times a week
○ Once a week

Since (mm/yyyy) ☐☐ / ☐☐☐☐

Stop Date* (mm/yyyy) ☐☐ / ☐☐☐☐

Did you ever smoke or currently smoke cigarettes?

○ Never

○ Currently
○ I Used To

smoke ☐☐.☐ packs

Since (mm/yyyy) ☐☐ / ☐☐☐☐

Stop Date* (mm/yyyy) ☐☐ / ☐☐☐☐

Have you ever used and drugs such as marijuana, cocaine, stimulants, sedatives or other illicit drugs?

Drugs:	○ marijuana	○ cocaine	○ stimulants	○ sedatives	○ narcotics	○ diet pills	○ other
Dosages:	○ regularly	○ regularly	○ regularly	○ regularly	○ regularly	○ regularly	○ regularly
	○ occasionally	○ occasionally	○ occasionally	○ occasionally	○ occasionally	○ occasionally	○ occasionally
	○ unknown	○ unknown	○ unknown	○ unknown	○ unknown	○ unknown	○ unknown
Started* (yyyy)	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐
Stopped* (yyyy)	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐

*write "curr" or "__/curr" if drug is still in use. If unknown, code "?" (i.e. ??/200?) If not applicable, leave empty. (i.e no stop date)

5865378541

Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

General Demographic Information

Are you currently covered by medical insurance or a health plan? ○ Yes ○ No

If **YES**, which plan? ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Do you participate in the Women, Infants and Children (WIC) program? ○ Yes ○ No

Do you participate in any other public assistance programs? ○ Yes ○ No

If **YES**, which program? ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Do you live alone? ○ Yes ○ No

If **NO**, who do you live with (bubble all that apply)?

○ Mother ○ Father ○ Significant Other ○ Brother(s) ○ Sister(s) ○ Aunt / Uncle
○ Cousin(s) ○ Friend ○ Grandparent(s) ○ Roomate ○ Other

What is your current marital status? ○ Single ○ Married ○ Divorced ○ Widowed
○ Other: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

What is your ethnicity? ○ Hispanic ○ Non-Hispanic

What is the ethnicity of the biological father of the baby? ○ Hispanic ○ Non-Hispanic ○ Unknown ○ Abstain

What is your race?
○ American Indian or Alaska Native
○ Native Hawaiian or Other Pacific Islander
○ White / Caucasian
○ Asian
○ Black or African American
○ Other

What is the race of the biological father of the baby?
○ American Indian or Alaska Native
○ Native Hawaiian or Other Pacific Islander
○ White / Caucasian
○ Asian
○ Black or African American
○ Abstain
○ Unknown
○ Other

What is your highest level of education completed? ☐☐ Years 0-6 – Primary School 7-12 – Secondary

Contact Information

Home Phone Number
☐☐☐ - ☐☐☐ - ☐☐☐☐

Alternate Phone Number
☐☐☐ - ☐☐☐ - ☐☐☐☐

Address
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐ ☐☐ ☐☐☐☐☐ - ☐☐☐☐

Emergency Contact Phone Number
☐☐☐ - ☐☐☐ - ☐☐☐☐

Relationship
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Name
☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Other

○ Yes ○ No Relatives With Patient? Who? ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

ID# ☐☐☐ **Visit Date** (mm/dd/yy) ☐☐ / ☐☐ / ☐☐ **Visit** ○ 1 ○ 2 ○ 3

APPENDIX 2

MATERNAL ANTHROPOMETRIC DATA COLLECTION TELEFORM

3797621993

Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study -Anthropometric Measurements

Height

1. ☐☐☐.☐ centimeters 2. ☐☐☐.☐ centimeters 3. ☐☐☐.☐ centimeters

Weight

1. ☐☐☐.☐ ○ Kilograms ○ Pounds 2. ☐☐☐.☐ ○ Kilograms ○ Pounds 3. ☐☐☐.☐ ○ Kilograms ○ Pounds

Blood Pressure

☐☐☐ / ☐☐☐ mmHg ☐☐ / ☐☐ / ☐☐ BP Taken on the date

Heel Ultrasound

	1.	2.	3.
BMD:	☐.☐☐☐	☐.☐☐☐	☐.☐☐☐
QUI:	☐☐☐.☐	☐☐☐.☐	☐☐☐.☐
BUA:	☐☐☐.☐	☐☐☐.☐	☐☐☐.☐
SOS:	☐☐☐☐.☐	☐☐☐☐.☐	☐☐☐☐.☐
T-Score:	☐☐.☐☐	☐☐.☐☐	☐☐.☐☐

Comments

○ Bubble here if comments section must be reviewed

ID# ☐☐☐ **Visit Date** mm/dd/yy ☐☐ / ☐☐ / ☐☐ **Visit** ○ 1 ○ 2 ○ 3

03/26/2007
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APPENDIX 3

MATERNAL PHYSICAL ACTIVITY RECALL DATA COLLECTION TELEFORM

3476080233

Maternal / Fetal Bone Health In Pregnant Adolescents
Physical Activity Questionnaire

Physical Activity Log For The Date of **Record Date** mm/dd/yy ☐☐ / ☐☐ / ☐☐

Start Time	Stop Time	Activity	VL	L	M	H	Categories & Activities
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	1 - Eating 110 - Meal;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	120 - Snack; 130 - Cooking
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	2 - Sleeping/Bathing
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	210 - Sleeping; 220 - Resting;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	230 - Shower / Bath
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	3 - Transportation 310 - Ride in car / bus;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	320 - Travel by walking; 330 - Travel by bike
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	4 - Work/School 410 - Job;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	420 - Homework;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	5 - Spare Time
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	510 - Household chores; 520 - Watch TV;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	530 - Movies / Concerts; 540 - Listen to music;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	550 - Talk on the phone; 560 - Hang around;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	570 - Shopping; 580 - Hobby (list);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	590 - Other (list);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	6 - Play/Recreation
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	610 - Bicycle(easy); 620 - Bicycle(fast);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	630 - Walk(slowly); 640 - Walk(fast);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	650 - Jog/Run; 660 - Dance(fun);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	670 - Aerobic dance; 680 - Swim(fun);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	690 - Swim laps; 691 - Skateboard;
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	692 - Lift weights; 693 - Organized sport(slow);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	694 - Organized sport(fast); 695 - Indivual sport(slow);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	696 - Individual sport(fast);
☐☐:☐0	☐☐:☐0	☐☐☐	○	○	○	○	

ID# ☐☐☐ **Visit Date** mm/dd/yy ☐☐ / ☐☐ / ☐☐ **Visit** ○ 1 ○ 2 ○ 3

02/12/2007
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APPENDIX 4

CALCIUM FOOD FREQUENCY QUESTIONNAIRE (FFQ) DATA COLLECTION TELEFORM

5121224287

Teen Bone Study
Calcium Food Frequency Questionaire

Milk ○ Skim ○ Whole ○ 2% ○ 1%	D	W	M				1 cup	Pudding made with milk	D	W	M				½ cup
Chocolate Milk	D	W	M				1 cup	Orange, fresh	D	W	M				1 med
Nonfat dry milk powder	D	W	M				½ cup	Almonds	D	W	M				1/3 cup
Cheese Spread or Food	D	W	M				1 oz	Raisins or dates	D	W	M				¼ cup
Cheese sauce	D	W	M				¼ cup	Peanut Butter	D	W	M				2 tbsp
American cheese plain	D	W	M				1 slice	Cake	D	W	M				1 slice
Grilled cheese sandwich	D	W	M				1 each	Cookie:	D	W	M				1 each
Cottage cheese	D	W	M				1 cup	Cookie:	D	W	M				1 each
String cheese	D	W	M				1 stick	Candy bar:	D	W	M				1 each
Blue cheese	D	W	M				½ cup	Candy bar:	D	W	M				1 each
Cheddar cheese	D	W	M				1 oz	Eggnog	D	W	M				1 cup
Swiss cheese	D	W	M				1 oz	Hot cocoa mix with milk	D	W	M				1 pkt
Mozzarella cheese	D	W	M				1 oz	OJ with calcium	D	W	M				1 cup
Ice cream or ice milk	D	W	M				1 cup	Sunny Delight with calcium	D	W	M				1 cup
Fudgesicle	D	W	M				1 each	Macaroni cheese - homemade	D	W	M				½ cup
Ice cream sandwich or bar	D	W	M				1 bar	Macaroni cheese - mix	D	W	M				½ cup
Frozen yogurt	D	W	M				1 cup	Pizza	D	W	M				1 slice
Yogurt flavored or plain	D	W	M				1 cup	Lasagna or pasta with cheese	D	W	M				1 cup
Fast food milkshake	D	W	M				12 oz	Stuffed pasta with cheese	D	W	M				1 cup
Custard pie	D	W	M				1 slice	Spaghetti with tomato sauce	D	W	M				1 cup

D = Daily, W = Weekly, M = Monthly.

ID# ☐☐☐ **Visit Date** mm/dd/yy ☐☐/☐☐/☐☐ **Visit** ○1 ○2 ○3

7314224283

Teen Bone Study
Calcium Food Frequency Questionaire

Ravioli canned	D	W	M			.		1 cup	Biscuit or combread	D	W	M			.		1 med
Tomato or cream soup with milk	D	W	M			.		1 cup	Bread white or wheat	D	W	M			.		1 slice
Cheeseburger	D	W	M			.		1 each	Rolls buns	D	W	M			.		½ ea.
Hamburger	D	W	M			.		1 each	Breakfast Great Starts	D	W	M			.		1 pkg
Enchilada / Burrito meat & cheese	D	W	M			.		1 each	Egg McMuffin	D	W	M			.		1 ea.
Enchilada / Burrito beans & cheese	D	W	M			.		1 each	Eggs or egg substitute	D	W	M			.		1 or ¼ c.
Taco w/ meat & cheese	D	W	M			.		1 each	Omelet or egg w/ cheese	D	W	M			.		2 eggs
Shrimp	D	W	M			.		3 oz	Doughnuts	D	W	M			.		1 ea.
Canned salmon w/ bones	D	W	M			.		½ cup	Cold cereal	D	W	M			.		½ cup
Tuna sandwich / casserole	D	W	M			.		1 each	Poptarts / toaster strudel	D	W	M			.		1 ea.
Broccoli-raw	D	W	M			.		1 cup	Hot cereal w/ milk	D	W	M			.		½ cup
Broccoli-cooked	D	W	M			.		1 cup	Hot cereal w/ water	D	W	M			.		½ cup
Greens (collard,spinach)	D	W	M			.		½ cup	Soy products eg. Veggie burger	D	W	M			.		1 ea.
Baked beans	D	W	M			.		1 cup	Multivitamin	D	W	M			.		1 tab
Kidney or lima beans	D	W	M			.		1 cup		D	W	M			.		
Scalloped potatoes	D	W	M			.		1 cup		D	W	M			.		
Tofu	D	W	M			.		½ cup		D	W	M			.		
Waffle or pancake-home	D	W	M			.		1 lg.		D	W	M			.		
Waffle or pancake-mix	D	W	M			.		1 lg.		D	W	M			.		
Muffin	D	W	M			.		1 med		D	W	M			.		

D = Daily, W = Weekly, M = Monthly.

ID# | **Visit Date** mm/dd/yy / / | **Visit** 1 2 3

APPENDIX 5

PRENATAL SUPPLEMENT SURVEY DATA COLLECTION TELEFORM

4202461278

Teen Bone Study
Prenatal Supplements

What prenatal supplements did you midwife / nurse recommend? How much and how often and are you taking them? Please also indicate which supplements you are not taking. Which supplments are you currently taking (indicate all supplements)?

Supplement	Prescribed Freq.	Actual Frequency
Prenatal Multivitamin Midwife Recommended	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never
Iron Midwife Recommended	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never
Calcium Midwife Recommended	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never
Vitamin D Midwife Recommended supplementation start date	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never
Other Midwife Recommended	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never
Other Midwife Recommended	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never	Pills Every Day 2-5 times a week Once a week Occasionally Very rarely Skip / Never

ID# Visit Date mm/dd/yy / / Visit 1 2 3

4502461271

Teen Bone Study
Prenatal Supplements

What are the reasons for taking or not taking your pills?

How do the pills make you feel?

What do you think these pills will do for you and your baby?

Can you think of anything that might help you take your pills?

How often have you refilled your prescription? ○ Never ○ 1 X ○ 2 X ○ 3 X ○ 4 X (or more) ○ OTC

Are you craving anything other than food? ○ Pica.

ID# Visit Date mm/dd/yy / / Visit ○ 1 ○ 2 ○ 3

APPENDIX 6

FETAL ULTRASOUND DATA COLLECTION TELEFORM

1188030214

Teen Bone Study
Obstetrics Report

General Evaluation

Date: __ __ / __ __ / __ __ __ __

Placenta: ○ Anterior ○ Posterior ○ Fundal ○ Previa

Amniotic F.V.: ○ Within normal limits ○ Increased ○ Decreased

Biometry

BPD:	. mm	G. Age:		weeks		days	
HC:	. mm	G. Age:		weeks		days	
AC:	. mm	G. Age:		weeks		days	
FL:	. mm	G. Age:		weeks		days	
Hum:	. mm	G. Age:		weeks		days	
Estimated FW:	gm		lbs	oz		% Tile	

Gestational Age

LMP:	weeks	days	EDD: / /
U/S Today:	weeks	days	EDD: / /
Best:	weeks	days	EDD: / /

Comments

Arm Area:	. mm	G. Age:	weeks	days
Arm Circumference:	. mm	G. Age:	weeks	days
Leg Area:	. mm	G. Age:	weeks	days
Leg Circumference:	. mm	G. Age:	weeks	days
Abdominal Wall Skin Thickness:	. mm	G. Age:	weeks	days

Impression

ID# Visit Date (mm/dd/yy) / / Visit ○ 1 ○ 2 ○ 3

06/20/2008
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APPENDIX 7

PLACENTAL CHARACTERISTICS DATA COLLECTION TELEFORM

2943591777

Teen Bone Study
Placenta Information Form

Placenta Info

Placenta Number: ☐☐☐☐ Patient ID: ☐☐

Processed in Rochester: ○ Yes ○ No

Placenta Delivery (Birth) Date:	☐☐/☐☐/☐☐ (mm/dd/yy)		Time: ☐☐:☐☐ ○ AM ○ PM
Placenta:	Weight: ☐☐☐ gm		Thickness at center: ☐☐.☐ inches
Placenta:	Height: ☐☐.☐ inches		Width: ☐☐.☐ inches
Cord:	Length: ☐☐.☐ inches	Position: ○ Normal ○ Abnormal	Cord Weight: ☐☐☐.☐ gm

Processing Info

Picked up by (initials): ☐☐☐ Received by (initials): ☐☐☐

	Date:	Time:	Time to processing:
Processed in Ithaca by: (Initials) ☐☐☐	☐☐/☐☐/☐☐ (mm/dd/yy)	☐☐:☐☐ ○ AM ○ PM	☐☐:☐☐ ○ AM ○ PM
Processed in Rochester by: (Initials) ☐☐☐	☐☐/☐☐/☐☐ (mm/dd/yy)	☐☐:☐☐ ○ AM ○ PM	☐☐:☐☐ ○ AM ○ PM

Photo taken by (initials): ☐☐☐ ○ Yes ○ No

Comments

○ Bubble here if comments are to be reviewed

ID# ☐☐☐

03/05/2009
Page 1 of 1

APPENDIX 8

INFANT BIRTH OUTCOMES DATA COLLECTION TELEFORM

9871024034

Teen Bone Study
Baby Data Form

Baby Data

Delivery Date: mm / dd / yy **Delivery Time:** : ○ AM ○ PM **Sex:** ○ Male ○ Female

Preterm: ○ Yes ○ No

Attending Provider: **Gravida:** **Para:**

GA at Delivery: W D **Revised EDC:** / / mm/dd/yy

Height: in **Prepregnant Wt:** lbs **Current Wt:** lbs **Weight gain:** lbs

Type of Delivery: ○ SVD ○ Assisted ○ 1st C-section ○ 2nd C-section ○ V-BAC

Antepartum Complications:

Duration of labor: Stage 1 hr min **Total Labor Time** hr min

Stage 2 hr min

Stage 3 hr min

Delivery Complications:

PE/PIH: ○ Yes ○ No

Weight at birth: gms **Length at birth:** . cms **Head circumference:** . cms

Bubbles: ○ SGA/IUGR ○ LGA

APGAR scores: 1 min. 5 min. 10 min.

Presence of meconium: amniotic fluid: ○ Yes ○ No meconium aspiration: ○ Yes ○ No

Discharged to: ○ Newborn Nursery ○ SC Nursery ○ Strong NICU ○ Other (specify below)

Medication		Frequency	Date (mm/dd/yy)
○ Mom ○ Baby	Dose / Unit		Start / Stop
○ Mom ○ Baby	Dose / Unit		Start / Stop
○ Mom ○ Baby	Dose / Unit		Start / Stop
○ Mom ○ Baby	Dose / Unit		Start / Stop

Office Use Only

ID#

03/05/2009
Page 1 of 1

APPENDIX 9

TEEN BONE STUDY PARTICIPANT CONSENT FORM

HIGHLAND
HOSPITAL

Department of Obstetrics and Gynecology
Rochester Adolescent Maternity Program (R.A.M.P.)

905 Culver Road
Suite B
Rochester, NY 14609
585.275.2962
585.482.1666 fax
www.highlandhospital.org

Medicine of the Highest Order

An Affiliate of the
University of Rochester Medical Center

Consent Form

Study Title: **Maternal / Fetal Bone Health in Pregnant Adolescents**
Principal Investigators: **Thomas McNanley, M.D., Elizabeth Cooper, CNM, EdD, FACNM**
Co-Investigator: **Kimberly O'Brien, Ph.D.**

Introduction

This consent form describes a research study and what you may expect if you decide to participate. You are encouraged to read this consent form carefully and to ask the person who presents it any further questions you may have before making your decision whether or not to participate.

This study is being conducted by Thomas McNanley, MD, and Beth Cooper, CNM, EdD and Kimberly O'Brien, PhD, of the University of Rochester's Department of Obstetrics and Gynecology and Highland Hospital's Department of Obstetrics and Gynecology. Kimberly O'Brien is also on the faculty at Cornell University.

You are being asked to participate in this study because you are pregnant and 18 years of age or younger.

Purpose of Study

The purpose of the study is to learn more about bone health in pregnant teens and about how babies' bones grow before they are born. Calcium and vitamin D are found in foods that you eat and are needed for healthy bones. Teenage girls build most of their bone by the time they are 17-18 years old. When teenagers become pregnant they may need extra calcium. Too little calcium and vitamin D in the diet might cause teenage girls to lose bone. It might also slow the growth of their baby's bones.

You can volunteer for this study if you:

- Are 18 years old or younger
- Are less than 30 weeks pregnant
- Do not have high blood sugar (diabetes)
- Do not have HIV infection
- Do not have problems eating and digesting your food
- Do not have an eating disorder (such as anorexia)

Description of Study Procedures

If you decide to participate in this study you will be asked some questions to see how healthy you are. We will also look in your medical chart to learn more about how healthy you are and to learn more about your pregnancy. We would like you to come to Highland Hospital up to three times during your pregnancy, with visits usually being spaced at least four weeks apart so we can:

- Measure how tall you are and how much you weigh.
- Take a measurement of your blood pressure and heart rate.
- Measure the distance between your knee and your heel.
- Take a picture of your baby (called an ultrasound or sonogram). This test is safe to use during pregnancy.
- Ask you questions about what you eat and how you decide what to eat, and about how much you exercise.
- Ask you questions about how often you use drugs or alcohol.
- Measure how much bone you have in your body by putting your heel in a special ultrasound machine that will not hurt you or your baby.

Each visit may take up to two hours, so we will offer you some food and a drink during that time.

At one of these visits, when you are approximately **20 - 30 weeks pregnant**, we will also:

- Take a blood sample from your arm (about 4 teaspoons). Before the blood is taken, the nurse will ask you if you want some special cream to be put on your arm to make it hurt less. We will keep this blood sample until the entire sample collected has been used to measure nutrients.

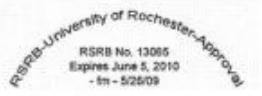
We will use this blood sample to measure:

- Hormones and proteins that your body needs when you are pregnant
- Levels of lead in the blood
- Levels of vitamin D in the blood

Lead is found in many homes in Rochester. If it gets into your body it can make you and your baby sick. We will call your doctor right away if you have too much lead in your blood. Your doctor can decide if you need medicine to get rid of this lead. If you have very high lead in your blood, you will not be able to continue in this study.

Second Part of Study (Optional)

If you would like to, you can also participate in the second part of the study on the day your baby is born. It is okay if you do not want to participate in this second study. You can still be in the first part of the study. If you want to participate in this second part, your medical chart will have a sticker put on it. The sticker will let the doctors and nurses know that they should call us after you go into labor and before your baby is born.



If you decide to participate in part 2, we will:

- Collect a sample of your blood (3 teaspoons). A blood sample is normally taken from your arm when you enter the hospital to have your baby and the sample we are collecting will be drawn at the same time. We will use this sample and the blood sample from the placental cord to measure hormones and proteins that your body needs when you are pregnant.
- Record the birth weight and birth length and other health information on your baby from your baby's medical chart.
- After a baby is born, the baby's afterbirth (called the placenta) comes out. The placenta is usually thrown away as waste. Before it is thrown away we will take some pieces of the placenta. This will help us to learn more about how your placenta sent nutrients to your baby while you were pregnant. We will also take some blood from the cord of the placenta (2-3 tsp.). This cord blood sample will tell us about the nutrients and hormones that your baby has when he/she is born. We will keep the blood samples until the entire sample has been used to measure nutrients.

We will use the blood sample we get from the placental cord blood to measure:

- Hormones and proteins that your baby needs for healthy bones
- Levels of lead in the blood
- Levels of vitamin D in the blood

If you have lead in your body some of this lead may be sent to your baby while you are pregnant. This lead can make your baby sick. If your baby has too much lead in their cord blood, we will call your baby's doctor right away. Your baby's doctor can decide if your baby needs medicine to get rid of this lead.

We would also like to measure how much bone you and your baby have in your bodies using a special test called a DXA. The DXA machine is located at Strong Memorial Hospital. We will try to schedule this test within 4 weeks of your baby's delivery and ask you to come to Strong Memorial Hospital with your baby for this final test.

The Dual energy X-ray absorptiometry (DXA) scan will measure the bone density of your whole body and of your lower spine. For the whole body scan, you will be asked to lie flat on your back on a table as the scanning machine moves above your body. From the scan of your whole body we will be able to see how much calcium you have in your entire body. We can compare this to the amount that is normally found in teenagers that are your age. This test will also measure the amount of fat and lean tissue in your body. For the scan of your lower back you will be asked to also lie on your back on the padded table, a large fabric covered foam cube will be placed under your lower legs so that you are in the best position to take the measure of you lower back area.

These DXA scans are like an X-ray, and these tests will take about 5-10 minutes to complete. It is safe to use if you are not pregnant. You should not have this test if you may be pregnant. Therefore, you will need to read and sign the "DXA waiver" and you may be tested for pregnancy before the DXA using a urine test.

The DXA test on your newborn will take approximately 2 minutes. Your baby will be wrapped in a blanket and lie on a padded table for the test. You can stay in the room with your baby while the test is conducted. To complete the entire visit for this study should take 40-60 minutes.

Number of Subjects

We plan to recruit a total of 300 adolescents to volunteer for this research study.

Risks of Participation

There are no known risks from the ultrasounds that we will take of your heel and of your baby. A total of 4 teaspoons of blood will be taken during pregnancy. A total of 7 teaspoons of blood will be taken if you volunteer for both studies. You may get a bruise and it may hurt a bit when the blood samples are taken. Some people feel lightheaded or faint when their blood is drawn. There is also a rare risk of infection. The samples of the placenta are collected after the baby is born and will not cause any risk. Use of the cream to numb you arm may cause irritation.

The DXA scans you will receive from getting the bone density test if you participate in part 2 of the study(the delivery part) involve radiation exposure which is less than a person would receive from a chest X-ray or a dental X-ray. The radiation amount is about what you would get in one day from the natural radiation around us. The amount of radiation your baby will receive from this test is also similar to what he/she receives from the natural background each day. We don't know whether any radiation dose is completely safe. The risk from the dose you and your baby will receive is considered low compared to other everyday risks.

Benefits of Participation

Screening for lead in your blood during pregnancy may be a benefit. High blood levels can be treated. We will tell you and your primary care provider if we find high lead levels. We will also test your blood for levels of vitamin D. Low vitamin D can be treated with supplements. If you are found to have low vitamin D levels we will tell you and share this information with your primary care provider. There is no health benefit provided by ultrasound screening. The ultrasound of your baby is only to tell us about the growth of your baby. However, if we found anything that made us worry that your baby was not healthy, we would tell you and your obstetrician right away.

Alternatives to Participation

You do not have to participate in this study if you do not want to. Your decision not to join this study will not affect the health care you receive at Highland Hospital or elsewhere.

Payments

Part 1:

For completing the **first visit** you will be given;

- A sonogram picture of your baby
- A small photo album

For completing the **second visit** you will be given;

- A sonogram picture of your baby
- A $10 Target or Walmart gift card (you can decide which store you prefer)

For completing the **third visit** you will be given;

- A sonogram picture of your baby
- A $15 Target or Walmart gift card (you can decide which store you prefer)

Part 2:

If you participate in part 2 when your baby is born we will take a digital picture of you with your baby, if you would like. The picture is a gift and will not be saved or used for other research purposes. You can have your picture taken in the hospital or during your post partum visit at RAMP.

On the day that you come into the GCRC at Strong Memorial Hospital for the DXA study you will receive $25.00 in cash.

Sponsor Support

The University of Rochester is receiving payment from the USDA for conducting this research study.

Confidentiality of Records and HIPAA Authorization

While we will make every effort to keep information we learn about you private, this cannot be guaranteed. Other people may need to see the information. While they normally protect the privacy of the information, they may not be required to do so by law. Results of the research may be presented at meetings or in publications, but your name will not be used.

The federal Health Insurance Portability and Accountability Act (HIPAA) requires us to get your permission to use health information about you that we either create or use as part of the research. This permission is called an Authorization. We will use;

- Demographic information (where you live, your phone number, etc.)
- Information on your height, weight and previous pregnancies
- Dietary information and information on supplement use over pregnancy
- Self reported drug and alcohol use and use of cigarettes
- Current use of medications and prescription drugs
- Diagnosis of any pregnancy complications or health problems

- Test results on hemoglobin and routine tests drawn across pregnancy
- The place where you were seen
- The name of your physician
- The medical records of your newborn

We will use your health information to conduct the study and to determine how your health status and other medical care issues that are happening during your pregnancy might be influencing the health of your bones and the growth of your developing baby. Health information is used to report results of research to sponsors and federal regulators. The health information collected may be audited to make sure we are following regulations, policies and study plans. URMC/Strong Health policies let you see and copy health information we have gathered for this research study after the study ends, but not until the study is completed. If you have never received a copy of the URMC/ Strong Health HIPAA Notice of Privacy Practices, please ask the investigator for one.

To meet regulations or for reasons related to this research, the study investigator may share a copy of this consent form and records that identify you with the following people. The University of Rochester; the Department of Health and Human Services; the United States Department of Agriculture, Cornell University, University of Rochester, Highland Hospital, and your primary care provider.

If you decide to take part, your Authorization for this study will not expire unless you cancel (revoke) it. The information collected during your participation will be kept indefinitely. You can always cancel this Authorization by writing to the study investigator. If you cancel your Authorization, you will also be removed from the study. However, standard medical care and any other benefits to which you are otherwise entitled will not be affected. Canceling you Authorization only affects uses and sharing of information after the study investigator gets your written request. Information gathered before then may need to be used and given to others.

As stated in the section on Voluntary Participation below, you can also refuse to sign this consent/Authorization and not be part of the study. You can also tell us you want to leave the study at any time without canceling the Authorization. By signing this consent form, you give us permission to use and/or share your health information as stated above.

Contact Persons

For more information concerning this research, please contact: Beth Cooper in the RAMP clinic at (585) 275-2962 or Kimberly O'Brien at (607) 255-3743.

If you have any questions about your rights as a research subject, you may contact the Human Subjects Protection Specialist at the University of Rochester Research Subjects Review Board, Box 315, 601 Elmwood Avenue, Rochester, NY 14642-8315, Telephone (585) 276-0005, for long-distance you may call toll-free, (877) 449-4441. You may also

contact the Cornell University Committee on Human Subjects (UCHS) at 607-255-5138, or via the web at; http://www.osp.cornell.edu/Compliance/UCHS/homepageUSHS.htm.

Voluntary Participation
Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without risking loss of present or future care you would otherwise expect to receive. In the event that you do withdraw from this study, the information you have already provided will be kept in a confidential manner.

Subject Consent

Second Part of Study
Please check one:

☐ I also agree to participate in the second part of the study when my baby is born.

☐ I do not agree to participate in the second part of the study when my baby is born.

Future Studies

The investigators from this study may want to contact you in the future regarding this study or to see if you and/or your child would be interested in participating in future studies. At this time you may decide whether or not you want to be contacted. If and when you are contacted you may decide if you and/or your child want to participate in any of the other studies and will sign another consent form to participate in those studies. Your decision regarding future contacts will not affect your participation in this study.

Please check one:

☐ Yes, I may be contacted in the future regarding this study **or** future studies.

☐ Yes, I may be contacted in the future for this study, but **not** for future studies.

☐ NO, I may not be contacted in the future.

Blood Samples

If it is okay with you we would also like to collect white blood cells from your blood so that we can screen for DNA and genes involved in how the body uses nutrients. The samples may be used to help identify genetic factors that influence how the body uses nutrients and to understand how these may be related to differences in bone loss or fetal bone growth. The samples will not be sold or used directly for the production of commercial products and will be kept in a locked lab. Reports about future research

done with the sample will NOT be kept in your health records, but the sample reports may be kept with study records or in other secure areas.

You can decide if you want your sample to be used for this type of research. Your decision can be changed at any time by notifying the study doctor in writing. Your decision about your sample will not affect your participation in this study or other studies.

Please check one:

☐ Yes, you may use my blood sample for the DNA studies described above.

☐ NO, you may not use my sample for the DNA studies described above.

Delivery Calcium Study

If you are interested, you can also participate in a smaller sub-study of the Bone Health study. This study is designed to learn how the calcium that you eat crosses the placenta to your baby. This smaller study would take place at Highland hospital when you go into labor to deliver your baby. It would involve one more blood sample from you, for which you would receive an additional $25 in cash.

Please check one:

☐ Yes, I am interested in hearing more about the Delivery Calcium study.

☐ NO, I am not interested in hearing about the Delivery Calcium study.

Signature/Dates

I have read (or have had read to me) the contents of this consent form and have been encouraged to ask questions. I have received answers to my questions. I agree to participate in this study. I have received a signed copy of this form for my records and future reference.

Study Subject: _____ Print Name

Study Subject: _____ Signature

_____ Date

Person Obtaining Consent

I have read this form to the subject and/or the parent/guardian has read this form. I will provide the subject and parent/guardian (if present) with a signed copy of this consent form. An explanation of the research was given and questions from the subject were solicited and answered to the subjects satisfaction. In my judgment, the subject has demonstrated comprehension of the information.

_____ Print Name and Title

_____ Signature

_____ Date

APPENDIX 10

ISOTOPE SUB-STUDY PARTICIPANT CONSENT FORM

Consent Form
Oral and Intravenous Tracer

Strong Health Midwifery Group
R.A.M.P. Program

Study Title: **Placental Calcium Flux in Pregnant Adolescents**

Principal Investigators: Thomas McNanley, M.D., Eva Pressman, M.D., Elizabeth Cooper, CNM, EdD, FACNM
Co-Investigator: Kimberly O'Brien, Ph.D.

Introduction

This consent form describes a research study and what you may expect if you decide to participate. You are encouraged to read this consent form carefully and to ask the person who presents it any further questions you may have before making your decision whether or not to participate.

This study is being conducted by Thomas McNanley, MD, Eva Pressman, M.D., and Beth Cooper, CNM, EdD and Kimberly O'Brien, PhD, of the University of Rochester's Department of Obstetrics and Gynecology and Highland Hospital's Department of Obstetrics and Gynecology. Kimberly O'Brien is also on the faculty at Cornell University.

You are being asked to participate in this study because you are pregnant and between 15 and 18 years of age. You are eligible for this study because you have already agreed to participate in the "Maternal/Fetal Bone Health in Adolescents" (Teen Bone Study) and the Delivery Study. In that study we are trying to learn how being pregnant affects your bone health, and how your baby's bones grow.

Purpose of Study

The purpose of the study is to learn more about how calcium that teenage girls eat when they are pregnant crosses the placenta to their baby.

You can volunteer for this study if you:

- Are between 15 and 18 years old
- Are already enrolled in the "Maternal/Fetal Bone Health in Adolescents" (Teen Bone) study
- Are already enrolled in the Delivery Study, so we are collecting your placenta after the birth of your baby

Background

We know that calcium that you eat crosses the placenta to reach your baby. However we do not know much about how this happens, or how much of the calcium you eat reaches your baby.

905 Culver Road
Rochester, New York 14609
Midwives: (585) 275-7892 • R.A.M.P. : (585) 275-2962 • Fax: (585) 482-1666

In this study, we will use two special forms of calcium to see how much and how quickly the calcium you eat reaches your baby. Calcium in our body and in our food has 4 different weights. Some of these forms of calcium exist at very low levels and are called stable isotopes. These special forms of calcium are already present in your body and in the foods you eat. They are not harmful to you or your baby. We can give these rare forms of calcium to you and then measure how much of this calcium is in your blood, how much is in the placenta, and how much is in the cord blood when your baby is born.

For this study, we will ask you to drink a small amount or water containing one of these forms of calcium (^{44}Ca) and have another form (^{42}Ca) injected through your IV. The calcium will be given to you when you are in the labor and delivery room, but before your baby is born.

In the Delivery Study, we are looking at what proteins in your placenta may help the calcium you eat reach your baby. In this study, we will use these special forms of calcium to trace the nutrient from when you eat it, across the placenta, and finally to your baby.

Description of Study Procedures

If you decide to participate in this study when you are admitted to the hospital to deliver your baby and your doctor thinks your baby will be born in the next few hours, we will ask you to drink a small amount (about 10 mL) of water that contains the special (^{44}Ca) form of calcium. When we ask you to drink the special calcium (^{44}Ca), we will also inject a different special form of calcium (^{42}Ca) into your blood. When you are admitted to the hospital to deliver your baby, you will have an IV put in by one of the medical staff. We can inject the calcium through your IV so you will not need another needle-stick. There will be 2.9 mg of calcium in the water you squirt into your mouth, and about 1 mg in the solution that we inject through your IV. This means we will be giving you a total of about 4 mg of calcium. This is a very small amount; a normal 8 oz. glass of milk contains about 300 mg of calcium in it.

Two hours after we give you this calcium, we will take a small amount of blood from your arm (about 1 teaspoon). We will use this blood sample to determine how much of the ^{42}Ca that we injected is present in your blood, and how much of the ^{44}Ca that you drank was actually absorbed from your stomach and intestines into your body.

Because you are part of the Delivery Study, we are already collecting your placenta and cord blood. We will also look for any of the special calcium in the cord blood and the placenta to see how much was transferred from your own blood to your baby.

After you deliver your baby, if your doctor agrees, we will ask to take another small amount of blood from your arm (about 1 teaspoon). This blood sample will allow us to see how quickly the Ca you were given is being used by your body to build your and your baby's bones. This blood sample is optional and you can say no if you don't feel up to it at the time.

Of course it is hard to know how you will be feeling on the day that you deliver your baby, and you can change your mind about participating in this study at any time.

We will only start this calcium study if your doctor agrees that your labor is progressing normally.

Number of Subjects
We plan to recruit a total of 30 adolescents to volunteer for this research study.

Risks of Participation
You may get a bruise and it may hurt a bit when the blood samples are taken. Some people feel lightheaded or faint when their blood is drawn. There is also a rare risk of infection from having blood taken. The samples of the placenta are collected after the baby is born and will not cause any risk.

Benefits of Participation
There are no direct benefits to you for participating in this study.

Alternatives to Participation
You do not have to participate in this study if you do not want to. Your decision not to join this study will not affect the health care you receive at Highland Hospital or elsewhere.

Payments
You will receive $25.00 in cash after the first blood collection is obtained and you will receive an additional $10.00 if you decide to give the second blood sample immediately after your baby is born..

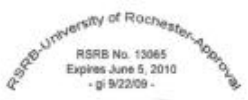
Sponsor Support
None claimed.

Confidentiality of Records and HIPAA Authorization

While we will make every effort to keep information we learn about you private, this cannot be guaranteed. Other people may need to see the information. While they normally protect the privacy of the information, they may not be required to do so by law. Results of the research may be presented at meetings or in publications, but your name will not be used.

The federal Health Insurance Portability and Accountability Act (HIPAA) requires us to get your permission to use health information about you that we either create or use as part of the research. This permission is called an Authorization. We will use;

- Demographic information (where you live, your phone number, etc.)
- Information on your height, weight and previous pregnancies
- Dietary information and information on supplement use over pregnancy
- Self reported drug and alcohol use and use of cigarettes
- Current use of medications and prescription drugs



- Diagnosis of any pregnancy complications or health problems
- Test results on hemoglobin and routine tests drawn across pregnancy
- The place where you were seen
- The name of your physician
- The medical records of your newborn

We will use your health information to conduct the study and to determine how your health status and other medical care issues that are happening during your pregnancy might be influencing the health of your bones and the growth of your developing baby. Health information is used to report results of research to sponsors and federal regulators. The health information collected may be audited to make sure we are following regulations, policies and study plans. URMC/Strong Health policies let you see and copy health information we have gathered for this research study after the study ends, but not until the study is completed. If you have never received a copy of the URMC/ Strong Health HIPAA Notice of Privacy Practices, please ask the investigator for one.

To meet regulations or for reasons related to this research, the study investigator may share a copy of this consent form and records that identify you with the following people. The University of Rochester; the Department of Health and Human Services; the United States Department of Agriculture, Cornell University, University of Rochester, Highland Hospital, and your primary care provider.

If you decide to take part, your Authorization for this study will not expire unless you cancel (revoke) it. The information collected during your participation will be kept indefinitely. You can always cancel this Authorization by writing to the study investigator. If you cancel your Authorization, you will also be removed from the study. However, standard medical care and any other benefits to which you are otherwise entitled will not be affected. Canceling you Authorization only affects uses and sharing of information after the study investigator gets your written request. Information gathered before then may need to be used and given to others.

As stated in the section on Voluntary Participation below, you can also refuse to sign this consent/Authorization and not be part of the study. You can also tell us you want to leave the study at any time without canceling the Authorization. By signing this consent form, you give us permission to use and/or share your health information as stated above.

Contact Persons

For more information concerning this research, please contact: Beth Cooper in the RAMP clinic at (585) 275-2962 or Kimberly O'Brien at (607) 255-3743.

If you have any questions about your rights as a research subject, or ay questions or complaints, you may contact the Human Subjects Protection Specialist at the University of Rochester Research Subjects Review Board, Box 315, 601 Elmwood Avenue, Rochester, NY 14642-8315, Telephone (585) 276-0005, for long-distance you may call toll-free, (877) 449-4441. You may also call this number if you cannot reach the research staff or wish to speak to someone else.

You may also contact the Cornell University Committee on Human Subjects (UCHS) at 607-255-5138, or via the web at:
http://www.osp.cornell.edu/Compliance/UCHS/homepageUSHS.htm.

Voluntary Participation

Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without risking loss of present or future care you would otherwise expect to receive. In the event that you do withdraw from this study, the information you have already provided will be kept in a confidential manner.

Future Studies

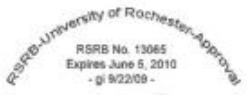
The investigators from this study may want to contact you in the future regarding this study or to see if you and/or your child would be interested in participating in future studies. At this time you may decide whether or not you want to be contacted. If and when you are contacted you may decide if you and/or your child want to participate in any of the other studies and will sign another consent form to participate in those studies. Your decision regarding future contacts will not affect your participation in this study.

Please check one:

☐ Yes, I may be contacted in the future regarding this study **or** future studies.

☐ Yes, I may be contacted in the future for this study, but **not** for future studies.

☐ NO, I may not be contacted in the future.



Signature/Dates

I have read (or have had read to me) the contents of this consent form and have been encouraged to ask questions. I have received answers to my questions. I agree to participate in this study. I have received a signed copy of this form for my records and future reference.

Study Subject: _____ Print Name

Study Subject: _____ Signature

_____ Date

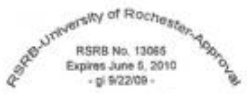
Person Obtaining Consent

I have read this form to the subject and/or the parent/guardian has read this form. I will provide the subject and parent/guardian (if present) with a signed copy of this consent form. An explanation of the research was given and questions from the subject were solicited and answered to the subjects satisfaction. In my judgment, the subject has demonstrated comprehension of the information.

_____ Print Name and Title

_____ Signature

_____ Date



APPENDIX 11

OSTEOPROTEGERIN (OPG) DIFFERS BY RACE AND IS RELATED TO INFANT BIRTH WEIGHT Z-SCORE IN PREGNANT ADOLESCENTS*

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ABSTRACT

Osteoprotegerin (OPG) is involved in the regulation of bone turnover but little is known about this protein during pregnancy or among neonates. We undertook a prospective longitudinal study to identify relationships between OPG, markers of bone turnover, and birth outcomes in 155 pregnant adolescents (13-18 y) and their newborns. Maternal blood samples were collected at mid-gestation and at delivery. Cord blood was obtained at delivery. Serum OPG, estradiol, and markers of bone formation (osteocalcin) and resorption (N-telopeptide) were assessed in all samples. Placental OPG expression was assessed in placental tissue obtained at delivery. Bone markers and OPG increased significantly from mid-gestation (26.0 ± 3.4 weeks) to delivery (39.3 ± 2.6 weeks). Neonatal OPG was significantly lower, but bone turnover markers significantly higher than maternal values at mid-gestation and at parturition ($p<0.001$). African American adolescents had higher concentrations of OPG than Caucasian adolescents at mid-gestation ($p = 0.01$) and delivery ($p = 0.04$). Gestational age and estradiol were also predictors of maternal OPG at mid-gestation and delivery. OPG concentrations in cord blood were correlated with maternal OPG concentrations and were negatively associated with infant birth weight Z-score ($p = 0.02$) and ponderal index ($p = 0.02$). In conclusion, maternal OPG concentrations increased across gestation and were significantly higher than neonatal OPG concentrations. Maternal and neonatal OPG concentrations were not associated with markers of bone turnover or placental OPG expression, but neonatal OPG was inversely associated with neonatal anthropometric measures. Additional research is needed to identify roles of OPG during pregnancy.

KEY WORDS

Adolescent Pregnancy, osteoprotegerin, Birth Weight, Bone

INTRODUCTION

Adolescent pregnancy is a significant public health problem in the United States. Approximately 410,000 adolescents gave birth in 2009.[1] When childbearing occurs prior to attainment of peak skeletal mass, there is a potential competition for nutrients between the adolescent and her developing fetus. Pregnant adolescents also have a greater risk for adverse birth outcomes including premature birth, low birth weight, and higher neonatal mortality when compared to adult women.[2]

Pregnancy is a state of high bone turnover as maternal calcium physiology responds to the calcium demands required to mineralize the fetal skeleton. Several studies have noted that markers of bone turnover increase throughout gestation.[3-6] Osteocalcin (OC) is the major non-collagenous protein found in bone and is commonly used as an index of bone formation. N-telopeptide (NTX) is a collagen cross-linking domain that is released into the circulation when bone is resorbed. Although overall bone metabolic activity increases during pregnancy, many reports have documented a net loss of maternal trabecular bone following gestation.[3,4,7] This pregnancy induced bone loss is greater in pregnant adolescents than among pregnant adults.[8,9]

In 1997, the Receptor Activator of Nuclear factor Kappa-β (RANK) / RANK-ligand (RANKL) / Osteoprotegerin (OPG) system was identified as a major regulator of the coordinated activity of osteoclasts and osteoblasts.[10] Interactions between RANK and RANK-L stimulate osteoclastogenesis and bone resorption. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and may protect bone mass by limiting RANK / RANK-L interactions.[11] At present, few studies have investigated OPG concentrations during pregnancy. Existing data are limited, and comprised of a few relatively small, cross-sectional studies,[12-14] that suggest that OPG concentrations increase from early pregnancy (15-18 weeks) to term.[12-14] It is known that the *in-*

utero environment plays a role in programming offspring's risk for chronic disease in adult life.[15,16] Several studies have linked infant size at birth with adult BMC and risk of osteoporosis.[17,18] Currently, the possible impact of variation in circulating OPG during pregnancy and in the fetus on bone turnover makers or other maternal or neonatal characteristics remains unexplored.

The objective of this research was to characterize the longitudinal changes and determinants of circulating OPG concentrations across gestation in a large cohort of pregnant adolescents and their neonates. We aimed to determine if OPG concentrations in neonatal and maternal circulation were significantly related, and if they were associated with other variables including bone turnover markers and placental OPG expression. We also investigated whether maternal or neonatal OPG concentrations were associated with infant birth weight Z-score and ponderal index.

METHODS

Study Participants:

A cohort of 155 pregnant adolescents (≤ 18 y) was recruited to participate in a prospective, longitudinal study of calcium and bone homeostasis. Adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY. Pregnant adolescents were between 12 - 30 weeks of gestation at entry into the study, were otherwise healthy, and were carrying a single fetus. Exclusion criteria included known medical complications or diseases such as HIV infection, diabetes, gestational hypertension and diagnosed eating disorders or malabsorption diseases. Informed written consent was obtained from all participants, and study procedures were approved by the Institutional Review Boards of the University of Rochester and Cornell University. Maternal race, ethnicity (Hispanic or non-Hispanic), and smoking history were self-reported. Infant race was classified as African American if the mother reported both herself and the father as African American; as Caucasian if the mother reported both herself and the father as Caucasian; or as biracial if the infant's maternal and paternal reported race differed. Infant ethnicity was classified similarly as either: Hispanic, non-Hispanic, or multi-ethnic. Age at menarche was self-reported, and gynecological age was calculated as the number of years elapsed from menarche to conception. At birth, infant weight and length were recorded by clinical staff using standard procedures and neonatal ponderal index (kg/m^3) was calculated using the length and weight measures. Birth weight Z-scores were generated using published curves specific to infant sex and gestational age at birth.[19] Upon delivery, the placenta was collected and weighed to the nearest 0.1 g.

Blood Sample Collection and Analyses:

A maternal blood sample (10 mL) was collected at mid-gestation by clinical staff during a routine prenatal visit. Because most visits occurred mid-day, participants were not counseled to fast before blood collection. At delivery, a second, non-fasting maternal blood sample and a 10 mL cord blood sample were collected. All samples were allowed to clot at room temperature before serum was separated and stored at -80^0 C until analysis. All biochemical analyses were obtained using these serum samples.

Maternal and cord blood NTX concentrations were measured in serum by ELISA (Ostex International, Seattle, WA) at the University of Rochester CLIA certified laboratory. Osteocalcin was measured at Yale University in the laboratory of Dr. Caren Gundberg using a radioimmunoassay that recognizes both the intact and major proteolytic product, as previously published[20] (intra-assay and inter-assay coefficient of variation (CV) were 3.8% and 7.1%, respectively). Serum OPG was measured using a commercially available ELISA (DY805: R&D Systems, Minneapolis, MN; inter- and intra-assay CV were 5.5% and 6.5%, respectively) and estradiol was analyzed using a commercially available ELISA from Alpco Diagnostics (Salem, NH; inter- and intra-assay CV were 4.2% and 10.1%, respectively). Leptin was measured using a commercially available ELISA from Millipore (Billerica, MA; inter- and intra-assay CV were 4.5% and 8.3%, respectively).

Placental Collection and Western Blot:

Five, one-square-inch placental tissue samples were collected from various locations throughout the entire placenta in order to obtain as representative a sampling as possible. Maternal and fetal membranes were removed and tissue samples were pooled, homogenized, and frozen at -80^0 C until analysis. Protein lysates were generated using a Polytron PT3100 tissue

homogenizer (Kinematica, Inc., Lucerne, Switzerland) at 5,000 rpm in a hypertonic lysis buffer containing protease inhibitor. Homogenized tissue was centrifuged at 13,000 rpm for 20 minutes at 4^0 C. Protein concentrations in the supernatant were determined with the Bio-Rad assay (Bio-Rad, Hercules, CA). Lysates were frozen at -80^0 C in SDS sample buffer until analysis.

Relative placental OPG expression was determined via Western blot. Lysates were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked in Odyssey Blocking Buffer (Li-Cor BioSciences, Lincoln, NB) for 1-h at room temperature and then probed with Goat-anti-OPG (AF805: R&D Systems, Minneapolis, MN) at a concentration of 0.3 µg/mL and Rabbit-anti-β-actin (AbCam, Cambridge, MA) at 6.7 ng/mL overnight at 4^0 C. Fluorescent secondary antibodies were used: Donkey-anti-Goat 800 at a concentration of 0.17 µg/mL (Li-Cor BioSciences), and Chicken-anti-Rabbit 647 at 0.13 µg/mL (Invitrogen, Carlsbad, CA). All antibodies were made in a solution of Odyssey blocking buffer diluted 1:1 in a phospho-buffered saline (PBS) solution. The OPG and β-actin bands were quantified using the Odyssey infrared imaging system (Li-Cor Biosciences), and the ratio of OPG to β-actin calculated. Intra-membrane variation was controlled for by normalizing the OPG: β-actin ratio of individual placentas to a standard control placental sample that was run on every gel. This standard placental control lysate was prepared as described above using tissue collected from a healthy adult woman delivering at term.

Statistical Analyses:

Statistical analyses were undertaken using SAS 9.2 and JMP 8.0 (SAS Institute Inc, Cary, NC). Results are reported as the mean ± standard deviation (SD) unless otherwise noted. Paired t-tests or non-parametric tests were used to assess changes in biochemical markers within subjects from mid-gestation to delivery. Independent t-tests or ANOVA were used to determine

if normally distributed variables differed by race, and the Wilcoxon Rank sum test was utilized for nonparametric data. The Shapiro Wilks test was used to assess normality of variables. Simple and multiple linear regression were used to assess statistical determinants of OPG at mid-gestation and delivery. Non-normally distributed variables were log transformed as necessary to ensure normality of models' residuals. In multiple linear regressions, interaction terms were evaluated, but none were significant and thus are not reported. When modeling determinants of mid-gestation OPG concentrations, one participant's data were excluded because her OPG value was an outlier (over four standard deviations from the mean). Study findings were not impacted if this data point was included or excluded. Simple and multiple linear regression analyses were used to assess the relationships between OPG and infant outcomes. Sample size calculations were undertaken to determine the sample size necessary to appropriately characterize mean OPG concentrations and yield a minimal detectable difference equivalent to 25% of the previously reported standard deviation in OPG concentrations in pregnant women at term. Using these parameters, we required a sample size of 120 to yield a power of 80% at an alpha of 0.05.[14] The sample size we achieved in this study also provided us with 80% power ($\alpha = 0.05$) to detect a minimal detectable difference of 0.24 in birth weight Z-score and 0.10 kg/m^3 in ponderal index.[19,21] Data were considered significant if P values were <0.05. P values between 0.05 and 0.10 were considered trends.

RESULTS

Subject Characteristics

Characteristics of the study population as a whole, and among the group when stratified by race are presented in **Table 1**. Of those enrolled; 65% were African American and 35% were Caucasian. Two teens identified their race as American Indian. In order to collapse maternal race into a bivariate category and avoid loss of data from the two American Indian adolescents, these two adolescents were included within the African American cohort. None of the race-specific analyses differed if data were analyzed with these two adolescents combined with either the African American or Caucasian cohorts, and all study results remained significant if these two subjects were excluded. Maternal age at enrollment into the study ranged from 13.6 to 18.7 years and birth weight Z-score ranged from -2.58 to 2.09. Approximately 8.6% of teens delivered prematurely (<37 weeks); 12.0% delivered a low birth weight (LBW) infant (<2500 g), and 6.7% delivered a large for gestational age (LGA) infant (>4500 g). A significantly higher percentage of Caucasian participants self identified as Hispanic (55.6%) than did African American participants (6.9%; $p<0.01$). There were no differences in length of gestation, birth weight, birth weight Z-score, ponderal index, rate of preterm, LBW, or LGA by infant sex, or maternal race (**Table 1**).

Estradiol increased by 127% (3077 ± 1674 vs. 5802 ± 2880 pg/ml) over the 13.1 ± 4.3 week interval that elapsed from the mid-gestation to delivery blood collection. The observed gestational increase in estradiol did not differ by race or maternal age across the 13-18 y age range studied.

Relationships between circulating maternal OPG and markers of bone turnover

Mean maternal concentrations of OPG, NTX, and OC all increased significantly from mid-gestation to delivery ($p<0.001$) (**Fig 1**) and mid-gestation values were significantly correlated with those at delivery ($p<0.001$ for all; n = 111, $R^2 = 0.55$; n = 114, $R^2 = 0.10$; n = 120, $R^2 = 0.27$, respectively). Serum OC and NTX were highly correlated with each other at mid-gestation (26.0 ± 3.4 weeks: $p<0.001$, n = 137, $R^2 = 0.08$) and at delivery (39.3 ± 2.6 weeks: $p<0.001$, n = 113, $R^2 = 0.26$). Furthermore, the rate of increase in OC from mid-gestation to delivery (change in OC (ng/mL)/weeks elapsed) was highly correlated with rate of increase in NTX ($p = 0.002$, n = 84, $R^2 = 0.11$). While correlated, the relative percent increase in NTX was significantly greater than the increase observed for OC (63% vs. 38%; $p<0.001$, n = 105). OPG was not significantly associated with NTX or OC at any time point measured. The relative increase in OPG (54%) did not significantly differ from that observed for NTX or OC. Neither maternal age (range: 13.6 – 18.7 y), nor gynecological age (range: 1.2 – 10.7 y) were associated with markers of bone turnover or OPG at any time point measured. Osteocalcin was higher in Caucasian adolescents at delivery than in African American adolescents ($p = 0.023$), although the difference between the observed means was minimal (0.7 ng/mL) and the relative increase in OC from mid-gestation to delivery did not differ as a function of race. In contrast, no significant differences in NTX were evident between racial groups when examined as either absolute values or as percent change across gestation.

Variables associated with circulating maternal OPG

At mid-gestation, none of the following variables were related to circulating OPG concentrations: maternal weight, weight gain, leptin, chronological age, gynecological age, maternal height, pre-pregnancy weight or BMI. At mid-gestation, both estradiol ($p = 0.001$, n =

136, $R^2 = 0.08$) and gestational age at sampling ($p = 0.010$, $n = 139$, $R^2 = 0.05$) were significantly positively associated with OPG. Furthermore, OPG in African American adolescents was on average 506 pg/mL higher than the mean observed in Caucasian adolescents ($p = 0.007$, $n = 139$). When NTX, estradiol, race, and gestational age were all entered into a model of mid-gestation OPG, gestational age was no longer significant. The best model identified (using maternal mid-gestation NTX, estradiol and race as predictors) accounted for 20% of the variability ($p<0.001$) in mid-gestation OPG.

At delivery, circulating OPG concentrations were not significantly related to maternal weight, weight gain, delivery leptin concentrations, height, pre-pregnancy weight, chronological age, or gynecological age. Similar to observations evident at mid-gestation, delivery OPG concentrations were significantly and positively related to gestational age ($p = 0.013$, $n = 123$, $R^2 = 0.05$) and were also positively associated with pre-pregnancy BMI ($p = 0.036$, $n = 123$, $R^2 = 0.04$). The racial difference in OPG persisted, and was of similar magnitude (444 pg/mL, $p = 0.044$) to that observed at mid-gestation. The relationship between estradiol and OPG at delivery approached significance ($p = 0.053$, $n = 122$, $R^2 = 0.03$), and was thus included as a covariate in the initial model of maternal OPG at delivery. The best model identified to predict OPG at delivery included estradiol, gestational age, and race ($p = 0.010$), but this model predicted only 7% of the variation in maternal OPG.

Neonatal OPG, OC, and NTX

NTX and OC were significantly higher in cord blood than in maternal blood at delivery ($p<0.001$). In contrast, OPG was significantly lower in cord vs. maternal blood at delivery ($p<0.001$) (**Fig 1**). Cord blood OC tended to correlate with maternal OC at mid-gestation ($p = 0.05$, $n = 100$, $R^2 = 0.04$), but not at delivery. NTX in cord blood was not associated with

maternal levels at any time point or with cord OC or OPG concentrations. In contrast, cord OPG concentrations were highly correlated with maternal OPG concentrations at mid-gestation ($p<0.010$, $n = 99$, $R^2 = 0.07$) and at delivery ($p = 0.001$, $n = 106$, $R^2 = 0.06$). Neonatal OPG was not associated with maternal or infant race, infant sex, estradiol, pre-pregnancy BMI, gestational age at delivery, or any other study variable collected.

Relationships between neonatal OPG concentrations and infant anthropometrics at birth

Neonatal OPG was significantly inversely correlated with both birth weight Z-score ($p = 0.029$; **Fig 2A**) and ponderal index (kg/m^3) ($p = 0.014$; **Fig 2B**). As expected, gestational age explained 37% of the variation in birth weight. Neonatal OPG alone was not significantly associated with infant birth weight ($p = 0.18$). However, when neonatal OPG was added as a covariate along with gestational age, OPG became a significant predictor of birth weight (parameter estimate $p = 0.020$, $n = 113$), and explained an additional 3% of the variation in birth weight. Similarly, gestational age at delivery explained 6% of the variability in ponderal index at birth. When neonatal OPG was added as a covariate to the model for ponderal index, the parameter estimate for OPG remained significant ($p = 0.006$) and explained an additional 2% of the variation in infant ponderal index.

The difference in average birth weight Z-score between the highest and lowest quartiles of cord blood OPG was 0.347. This difference represents a 192 g higher mean birth weight between the lowest vs. highest quartile of neonatal OPG. The difference in ponderal index between the lowest and highest quartiles of neonatal OPG was 1.72 kg/m^3. Overall, the mean ponderal index of infants in the highest quartile of neonatal OPG was significantly lower ($p<0.001$) than the average ponderal index previously published from a large cohort ($n = 1,040$)

of term infants.[21] The observed relationships between OPG and ponderal index and between OPG and birth weight Z-scores were independent of maternal and neonatal race.

Placental OPG

Placental OPG expression (via western blot) was assessed in 68 placental samples; a representative blot is shown in **Fig 3**. Neither placental weight nor placental expression of OPG was significantly associated with maternal OPG at mid-gestation or at delivery or with any of the other study measures obtained.

DISCUSSION

In this group of pregnant adolescents and their neonates, we found that maternal serum OPG concentrations across gestation and at birth were not significantly associated with maternal or neonatal bone turnover markers. Significant differences in maternal OPG concentration as a function of race were evident at both mid-gestation and at parturition. Of interest, neonatal concentrations of OPG were significantly correlated with, but lower than maternal concentrations and were negatively associated with neonatal anthropometric variables.

Expression of OPG has previously been characterized in the human placenta, amnion, and choriodecidual tissue.[10,13,22] Because maternal circulating OPG concentrations have been found to decrease by 38% within 4 days post-partum,[14] it has been postulated that the placenta contributes to circulating OPG during pregnancy.[13] However, while we were able to detect placental expression of OPG, we found no significant associations between placental OPG expression and OPG concentrations in maternal circulation at mid-gestation or delivery, or in neonatal blood at term. In contrast, maternal and neonatal OPG concentrations were highly correlated. Because little is known about the transfer of this protein across the placenta, the relative contribution of maternal or placental OPG to fetal concentrations remains unknown.

A significant negative relationship was noted between neonatal umbilical cord OPG concentrations and both birth weight Z-score and ponderal index. This is the first time, to our knowledge, that such a relationship has been reported. Neither the observed relationships nor infant characteristics were significantly influenced by maternal or infant race. A study by Briana et al. found no significant association between cord OPG concentrations and birth weight, and no difference in OPG between appropriate for gestational age (AGA; n = 20) and IUGR (n = 20) infants,[23] but their relatively small sample size may have limited their ability to detect

associations. Our observed association between cord OPG concentrations and neonatal birth weight Z-score may hint at a role for OPG in neonatal accrual of body stores of fat, bone, or muscle. In a study of lean and obese children (ages 5 – 16 y), OPG was negatively associated with total and truncal body fat mass when correcting for pubertal stage and gender.[24] However, in our study, OPG in cord blood was not related to cord blood or maternal leptin concentrations. The lack of a relationship between neonatal OPG and other indices of maternal nutritional status (BMI, weight gain, etc.), suggests that the association between neonatal OPG and neonatal size at birth is independent of maternal BMI during pregnancy.

The *in-utero* environment (nutritional and environmental) is known to impact fetal skeletal development and mineralization,[17,25-27] and changes evident at birth may be sustained into childhood and adulthood.[28,29] Maternal under-nutrition or "constraint" *in-utero* may impact offspring's adult risk of osteoporosis as studies have linked size at birth with adult bone mass,[17] and shown that the *in-utero* environment mediates the impact of genetics on adult BMC and BMD.[30,31] We report a significant negative relationship between cord OPG and both birth weight Z-score and ponderal index. Elevated OPG concentrations were observed among neonates that were smaller at birth. Because OPG plays a well-characterized role in the coordinated balance of osteoblast/osteoclast activity;[10,11] the elevated neonatal OPG may potentially play a programming role in subsequent bone growth and mineralization. A limitation of this study was that post-natal neonatal DXA measures were not obtained. These were originally planned but lack of compliance with the scheduled appointments following pregnancy precluded these measures.

Possible associations between OPG concentrations and concurrent changes in bone metabolic activity were explored. While significant increases in bone turnover markers occurred

across pregnancy, no significant relationships were evident between serum OPG and either OC or NTX in these adolescents. These findings are consistent with earlier data in pregnant adult women.[13,14] The lack of association between OPG and markers of bone turnover may indicate that OPG is playing additional roles aside from regulation of bone turnover during pregnancy.

In this group of pregnant adolescents, maternal OPG concentrations were significantly associated with gestational age over the time interval in which the mid-gestation blood samples were obtained (week 20 to week 37). Additionally, in the cohort as a whole, and in paired analyses, OPG concentrations significantly increased from the mid-gestation measure to the measure obtained at delivery. Other studies have shown that OPG increases throughout pregnancy.[13,14] Previous data from adult women have reported that OPG increases from 38% to 86% in the third trimester.[4,14] Our finding of a 54.2% increase in OPG from 26.0 ± 3.4 weeks gestation to delivery (39.3 ± 2.6 weeks gestation) is consistent with these prior data. While relative changes in OPG across gestation are variable, the absolute value we observed in these pregnant adolescents is significantly higher than previously reported for pregnant adults; in which values ranged from 863 pg/mL at 36 weeks,[4] to between 132 pg/mL,[12] to 1400 pg/mL at delivery.[14] Currently, no standard reference materials for OPG exist, thus we cannot rule out methodological differences as the source of the elevated OPG concentrations evident in our adolescent population. Elevated OPG concentrations have previously been reported among other groups known to have alterations in bone turnover, namely osteoporotic and anorexic women.[32-34] In these groups, the elevated OPG has been attributed to OPG's compensatory bone sparing properties.[32-34] While the OPG concentrations in this adolescent cohort were higher than typically reported among adult women, OPG concentrations were not significantly related to either chronological nor gynecological age of the adolescent.

Maternal race and circulating estradiol were significant predictors of maternal OPG at mid-gestation and at delivery. Previous data have shown that estradiol induces OPG expression in osteoblastic cells in-vitro,[35,36] but an earlier in-vivo study failed to detect a relationship between estradiol and OPG in serum during pregnancy.[4] The much larger sample size of our population and the increased bone turnover that occurs in pregnant adolescents may have influenced our ability to detect the observed associations. In this group of pregnant adolescents, African American teens exhibited significantly higher OPG concentrations compared to the Caucasian adolescents. This is the first time to our knowledge that a racial difference in OPG has been reported in a pregnant or non-pregnant population. Significantly higher bone mass is known to be evident among African American vs. non-Hispanic Caucasian women.[37] Our observed differences in OPG are consistent with these racial differences in bone mass.

The role of OPG during pregnancy remains poorly understood. Despite the significant associations observed in our study, we were able to capture only a small percentage of the variability in OPG (20% at mid-gestation and 7% at delivery) using the available study measures. In addition to classically-described roles, OPG also serves as a decoy receptor for the apoptosis-inducing ligand (TRAIL),[38] and binds both TRAIL and RANKL with similar affinity.[39] During pregnancy, placental OPG expression may protect placental membranes from the actions of TRAIL to maintain membrane integrity until parturition.[22] In addition, OPG may also play a role in modulating inflammation as OPG expression has been found to be upregulated by inflammatory cytokines including IL-1 and TNF-α[40] as well as by lipopolysaccharide (LPS) in choriodecidua,[22] but down-regulated by TRAIL.[41] OPG's potential role in the inflammatory response to pregnancy may be a partial source of the racial differences in OPG detected in these adolescents. Differences in pro and anti-inflammatory cytokines have been found between

African American and Caucasian women during pregnancy and at parturition,[42,43] but we did not measure inflammatory cytokines in our study. These alternate and yet undiscovered roles of OPG may account for the some of the unexplained variability in the OPG concentrations we observed.

In conclusion, in pregnant adolescents OPG concentrations increased across gestation but were unrelated to concurrent changes in markers of bone turnover. African American adolescents had significantly higher circulating concentrations of OPG compared to Caucasian adolescents, in spite of similar concentrations of bone turnover markers. Neonatal OPG was lower than, but highly correlated with maternal OPG concentrations, and was significantly inversely associated with birth weight Z-score and ponderal index. Further research is needed to elucidate the mechanisms driving these relationships, and fully clarify the roles of OPG on fetal physiology and possible associations with neonatal bone health and subsequent risk of osteoporosis.

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STATEMENT OF INTEREST

None

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Table 1: Characteristics of the Pregnant Adolescents and their Neonates at Birth[1]

Subject Characteristics	All Subjects	African American Adolescents	Caucasian Adolescents
Total Recruitment	155	65.2% (101)	34.8% (54)
Age at Enrollment (y) (weeks gestation)	17.1 ± 1.1 years (155) 21.7 ± 5.5 weeks (155)	17.1 ± 1.2 (101) 22.0 ± 5.5 (101)	17.2 ± 0.9 (54) 21.1 ± 5.6 (54)
Hispanic Non-Hispanic	23.8 % (37) 76.1% (118)	6.9 % (7)[2] 93.1% (94)	55.6 % (30) 44.4 % (24)
Pre-Pregnancy BMI	24.7 ± 5.3 kg/m^2 (153)	24.5 ± 5.2 (99)	25.1 ± 5.6 (54)
Multiparous (Parity ≥ 1)	9.0% (155)	9.9% (101)	7.4% (54)
Gestational Age at Mid-gestation Blood Collection	26.0 ± 3.4 weeks (152)	26.1 ± 3.3 (99)	25.9 ± 3.5 (53)
Gestational Age at Delivery	39.3 ± 2.6 weeks (149)	39.1 ± 2.9 (97)	39.6 ± 1.9 (52)
Birth Weight	3202 ± 583 g (147)	3151 ± 588 (95)	3294 ± 567 (52)
Infant Sex	55.3% Male (83)	56.1% (55)	53.8% (28)
Birth Weight Z-score	-0.392 ± 0.904 (147)	-0.463 ± 0.884 (95)	-0.260 ± 0.933 (52)
Infant Ponderal Index	24.52 ± 2.10 kg/m^3 (141)	24.25 ± 3.00 (91)	25.00 ± 3.26 (50)
OPG at Mid-Gestation	1949 ± 740 pg/mL (138)	2129 ± 764 (89)[3]	1623 ± 571 (49)
OPG at delivery	2812 ± 1215 pg/mL (123)	2975 ± 1295 (78)[4]	2530 ± 1016 (45)

[1]Data are reported as the Mean ± SD, or percentage, with the sample size in parentheses.

[2]Significantly different than Caucasians: $p<0.01$.

[3]Significantly different than Caucasians: $p<0.001$.

[4]Significantly different than Caucasians: $p<0.05$.

Figure 1:

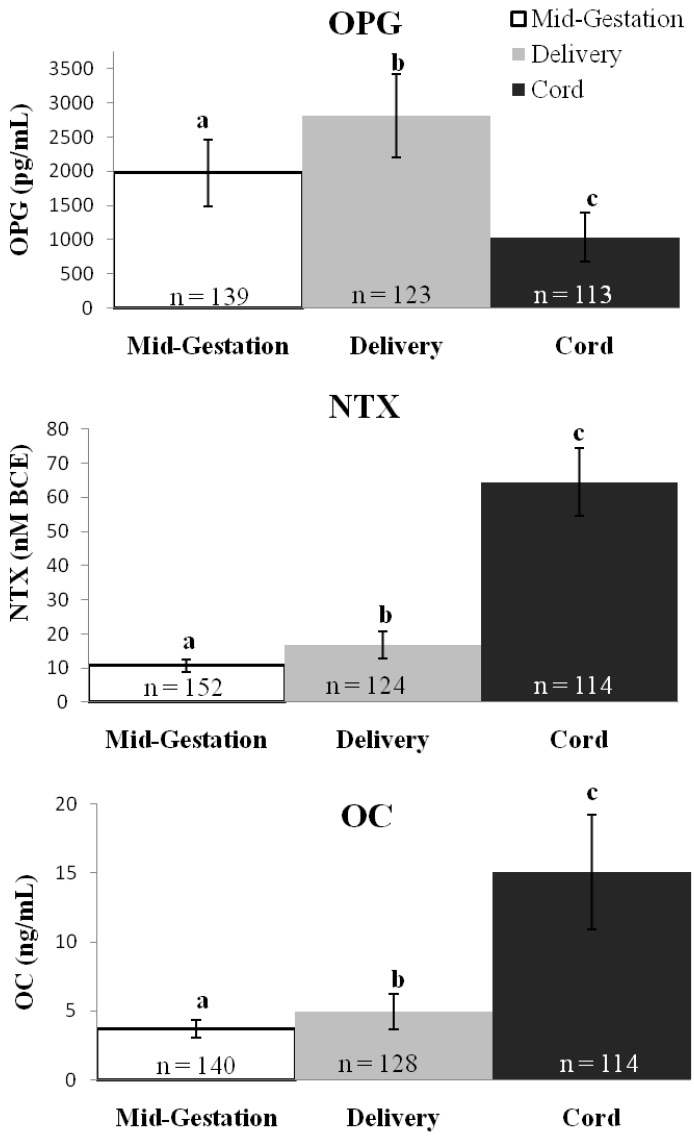

Figure 2:

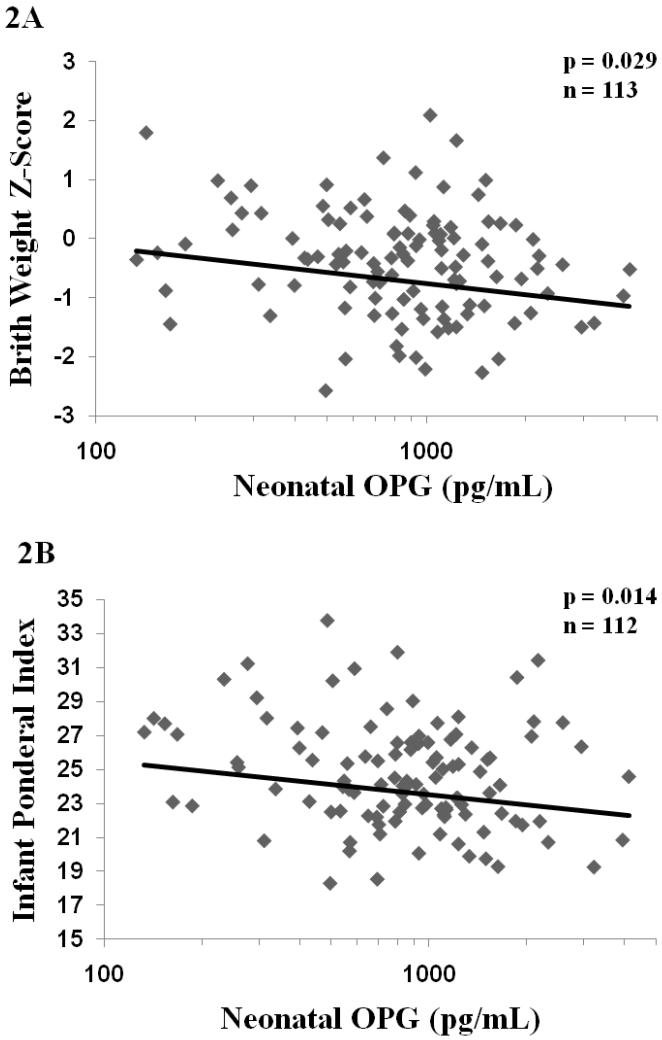
2A
p = 0.029
n = 113
Brith Weight Z-Score
3
2
1
0
-1
-2
-3
100
1000
Neonatal OPG (pg/mL)
2B
p = 0.014
n = 112
Infant Ponderal Index
35
33
31
29
27
25
23
21
19
17
15
100
1000
Neonatal OPG (pg/mL)

Figure 3:

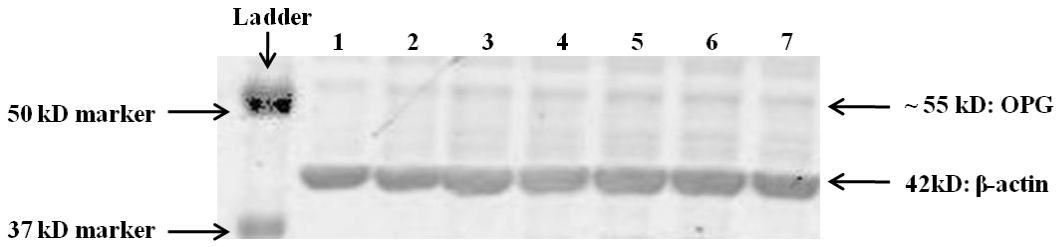
Ladder
1
2
3
4
5
6
7
50 kD marker
37 kD marker
~ 55 kD: OPG
42kD: β-actin

FIGURE LEGENDS

Figure 1:

Serum concentrations of osteoprotegerin (OPG), N-telopeptide (NTX), and osteocalcin (OC) were measured in a group of 155 pregnant adolescents at mid-gestation (26.1 ± 3.3 weeks) and at delivery (39.3 ± 2.6 weeks), and in cord blood obtained from neonates at birth. Different superscripts indicate group means significantly differed from each other ($p<0.0001$). In paired analyses, maternal concentrations of OPG ($p<0.0001$), NTX ($p<0.0001$), and OC ($p<0.0001$) were significantly higher at delivery compared to mid-gestation. Both NTX ($p<0.0001$) and OC ($p<0.0001$) were significantly higher in the neonate at birth compared to maternal concentrations at mid-gestation and delivery. In contrast OPG values in cord blood were significantly lower than those observed among pregnant adolescents at mid-gestation and delivery ($p<0.0001$).

Figure 2:

Neonatal serum OPG concentrations were measured in cord blood collected at delivery from a group of 113 pregnant adolescents. Neonatal OPG was significantly inversely related to birth weight Z-score ($p = 0.029$, $n = 113$, $R^2 = 0.05$) and infant ponderal index, calculated as kg/m^3 at delivery ($p = 0.014$, $n = 112$, $R^2 = 0.05$). The average birth weight in these neonates was 3261 ± 481 g.

Figure 3:

Protein lysates were generated from homogenized placental tissue samples collected from 68 pregnant adolescents at delivery, and OPG expression was analyzed via western blot. Placental OPG expression was not related to maternal OPG at mid-gestation ($p = 0.98$, $n = 59$) or at delivery ($p = 0.15$, $n = 62$). Shown is a representative western blot with individual placental lysate samples in lanes 1 - 7.